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A Study of Bacteriophage with Particular  
Reference to a Lysin-Producing Lactic Streptococcal  
Phage-Host System

by

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a

Thesis

Submitted for the

Degree of Doctor of Philosophy

in the

Faculty of Science

of the

University of Glasgow

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## ABSTRACT

Paired and multi-strain cultures containing phage-unrelated strains of lactic streptococci are widely used in phage control schemes in cheese factories. Infection of such cultures with phage will not normally markedly affect acid production since the phage-resistant strains will continue to produce acid and enable cheese manufacture to continue. The work contained in this Thesis relates to a previously undocumented phage for *Str. lactis* C2,  $\phi$ C2(W), which when added to paired and multi-strain cultures containing C2 inhibits acid production.

The phage was partially characterised with respect to host range, plaque morphology, latent period and burst size, particle morphology and the effect of temperature on replication. The phage which had a prolate polyhedral head had a short latent period (21 min) and was unusual in that it formed plaques surrounded by haloes. This latter property is indicative of a lysin-producing phage.

$\phi$ C2(W) lysates of C2 were shown to contain a lytic agent which lysed both phage-sensitive and phage-insensitive lactic streptococci. An assay method for measuring lytic activity is described.

The lytic agent was shown to be distinct from particulate phage. Centrifugation and acetone precipitation both reduced the level of phage (99 and 95% respectively) in  $\phi$ C2(W) lysates without markedly affecting lytic activity. Lytic activity, but not phage, was destroyed by mild heat treatment and was lost after chromatography on the dye ligand Green A. Lytic activity, but not phage, could also be washed out of  $\phi$ C2(W) lysates using ultrafiltration.



Lytic activity was demonstrated in cells of C2 infected with  $\phi$ C2(W) 10 min after infection and increased throughout the latent period reaching a high concentration at the rise period. Control cells or cells infected with  $\phi$ 712 did not demonstrate lytic activity. These results were interpreted as showing that the lytic activity in  $\phi$ C2(W) lysates was phage induced and that phage lysin could be involved in the release of phage from infected cells.

Experiments designed to determine the mechanism involved in the inhibition of acid production of paired and multi-strain cultures by  $\phi$ C2(W) yielded evidence that the inhibition was due to the lysis of the non-homologous strains by a lytic enzyme released from phage-infected cells of C2. The significance of these results to starter selection for cheese-making and measures for controlling the nascent phenomenon are discussed.

The lytic enzyme present in  $\phi$ C2(W) lysates was isolated using ion-exchange chromatography, gel filtration and ultrafiltration. The phage enzyme had an apparent pH optimum of 6.5 - 6.9. The partially purified lysin was very heat labile and was rapidly inactivated at temperatures  $> 47^{\circ}\text{C}$ . An apparent temperature optimum of  $37^{\circ}\text{C}$  was found and  $Q_{10}$  and  $E_a$  values over the range  $22 - 32^{\circ}\text{C}$  were 2.5 and  $69.2 \text{ kJ mole}^{-1}$  respectively.

Monovalent and divalent cations activated the lytic enzyme. Reduced sulphhydryl groups on the enzyme were required for activity. Unlike lysozyme (E.C.3.2.1.17) high concentrations of glucosamine (up to 50 mM) did not inhibit activity. Gel filtration gave a molecular weight of about 46,000 for the phage enzyme.

Strain dependent differences in sensitivity of Group N streptococci to phage lysin were found. Streptococci of Group D were also lysed. Strains of *Leucon. cremoris*, *Leucon. lactis*, *Leucon. dextranicum*, *Str. thermophilus*,

*Lact. bulgaricus*, *Lact. fermenti*, *E. coli*, *Micrococcus lysodeikticus* and *Str. dysgalactiae* were apparently resistant.

Cells which did not adsorb  $\phi$ C2(W) were also lysed by the phage enzyme suggesting that lysin and phage did not compete for the same adsorption sites on the streptococcal cell wall.

Examination of lysed cells, using the electron microscope, revealed that the phage enzyme removed the cell walls creating protoplasts.

Analysis of cell wall degradation products showed that the enzyme was a glycosidase. The analytical data were consistent with the phage lysin having the specificity of a N-acetylmuramidase.

The  $\phi$ C2(W) enzyme was compared with other Group N streptococcal phage lysins, and autolytic enzymes described for streptococci. It is argued that the  $\phi$ C2(W) enzyme is different from other streptococcal phage lysins and is unlikely to be an autolytic enzyme which has increased in concentration as a result of phage infection.

A preliminary study of factors affecting the sensitivity of *Str. lactis* C2 and some other lactic streptococci to  $\phi$ C2(W) lysin was undertaken. Log phase cells were more sensitive than those from stationary phase. Cells obtained from media containing glucose were lysed more rapidly than cells grown in media containing other carbohydrates.

Growth in media containing L-lysine, L-threonine or a mixture of both had little effect on sensitivity to lysin. Cells grown in media containing sub-lethal concentrations of penicillin G (0.1-0.15 IU/ml) demonstrated increased sensitivity to lysis by the lysin. Freeze-drying also increased the sensitivity of cells to lysis.

The basal medium used also affected the sensitivity of cells to lysis. Unlike lysozyme, the phage enzyme was not inhibited by 0.5 M sucrose. From the results of these and other experiments it is suggested that  $\phi$ C2(W) lysin may have value as an agent for removing the cell walls from Group N streptococci.

Variants of lactic streptococci were isolated which were virtually resistant to lysis by  $\phi$ C2(W) lysin. These variants were isolated using selective agar media containing either phage lysin or lysozyme. Contrary to expectations, lysin-resistant variants retained sensitivity to phage and therefore disproved the hypothesis of Reiter (1973) who speculated that lysin-resistant mutants should also be phage resistant. From these and other experiments, it is argued that  $\phi$ C2(W) lysin is not involved in the infection of C2 by phage nor in the release of phage from cells of C2 infected with  $\phi$ C2(W). The value of lysin production to a phage and the use of lysin-resistant mutants as cheese starters are discussed.

# AN INTRODUCTION TO THE LACTIC STREPTOCOCCI AND THEIR BACTERIOPHAGES

## LACTIC STREPTOCOCCI

The lactic streptococci used in the manufacture of fermented dairy products are members of the genus *Streptococcus*. They can be differentiated from other streptococci by their specific reaction with Group N antiserum and by their tolerance to temperature, salt and dyes (Jones, 1978).

The lactic group of the genus *Streptococcus* includes the species *Str. lactis* and *Str. cremoris* and a subspecies of *Str. lactis*, *Str. lactis* subspecies *diacetylactis* (Deibel and Seeley, 1974). However, the converse may be more correct, i.e. *Str. lactis* strains may be variants of *Str. diacetylactis* that are unable to ferment citric acid, since citrate permease - negative strains of *Str. diacetylactis* have been described (Lawrence, Thomas and Terzaghi, 1976).

Evidence is accumulating to suggest that *Str. cremoris*, *Str. lactis* and *Str. diacetylactis* form a phenotypically and genotypically continuous spectrum of variation (see Lawrence *et al.*, 1976; Lawrence and Thomas, 1979). The three species are similar in DNA base-composition (Knittel, 1965) indicating that they are closely related. It is now clear that the genus *Streptococcus* is closely related to the genera *Lactobacillus* and *Leuconstoc* (London, 1976). The designation 'lactic' was applied to this group by Sherman (1937) for mainly historical reasons, including the use of the term by Lister (1878) to describe a bacterium which we now know as *Str. lactis*. An alternative nomenclature is the term Group N streptococci since *Str. lactis*, *Str. cremoris* and *Str. diacetylactis* all possess the Group N antigen. Throughout this work the terms lactic and Group N streptococci will be used synonymously.

Members of the Group N streptococci can be differentiated to the species or subspecies level by schemes published by several authors

(Sandine, Elliker and Anderson, 1959; Reiter and Møller-Madsen, 1963; Reddy *et al.*, 1972; Diebel and Seeley, 1974; Jones, 1978).

Some biochemical properties of the lactic streptococci are shown in Table 1. Only one type of interpeptide bridge structure is shown for *Str. cremoris* instead of the two structures originally proposed by Schleifer and Kandler (1967). These workers found that two strains of *Str. cremoris*, strains 316a and 331a, contained a different interpeptide bridge namely 1-alanyl-threonine than other strains of *Str. lactis* and *Str. cremoris* studied. However, strains 316a and 331a possessed carbohydrate fermentation patterns which were not typical of *Str. cremoris*. Both strains fermented sucrose and maltose, characteristics which are not shared by typical strains of *Str. cremoris* (Jones, 1978; Garvie, 1980, pers. comm.). In a more recent publication Schleifer and Kandler (1972) have admitted that strains 316a and 331a were probably wrongly classified as *Str. cremoris*. This admission has apparently escaped Diebel and Seeley (1974) who still attribute two different types of peptidoglycan to *Str. cremoris*, based on the 1967 paper of Schleifer and Kandler.

The genetics of lactic streptococci have received increasing attention in recent years. Reports on the number and size of plasmids, or the presence of covalently closed circular DNA, indicate strain and species variation within the lactic streptococci (see Lawrence *et al.*, 1976). Progress is such that plasmid profiles have recently been used to prove strain similarity (Davies, Gasson and Underwood, 1981). There is now considerable evidence that certain industrially important characteristics of lactic streptococci, e.g. citrate, carbohydrate and protein metabolism and nisin production are plasmid mediated (Fuchs, Zajdel and Dobrzanski, 1975; Efsthathiou and McKay, 1976; Lawrence *et al.*, 1976; Lawrence and Thomas, 1979; Davies and Gasson, 1981).

Table 1

## Some properties of lactic streptococci

Species	Growth at			pH 9.2	Growth in media containing 4% NaCl	Growth in media containing 6.5% NaCl	Citrate utilised	Citratase present	Acetoin/diacetyl produced	Ammonia produced from arginine	Peptidoglycan type (2)	Crosslinkage in peptidoglycan	Acid produced from					
	10°C	39.5°C	45°C										Glucose	Maltose	Sucrose	Dextrin	Lactose	
<i>Str. lactis</i>	+	+	-	+	+	-	-	-	-	+	A4α	L-lys-D-asp	+	+	V	+	+	3
<i>Str. cremoris</i>	+	-	-	-	-	-	(1)	-	-	-	A4α	L-lys-D-asp	+	-(3)	-	-(3)	+	
<i>Str. diacetylactis</i>	+	+	-	+	+	-	+	+	+	+	ND	ND	+	+	-	+	+	

+ Growth

- No growth

V Strain dependant

Data based on information from Deibel and Seeley (1974), Jones (1978)

(1) Some strains utilise citrate in presence of carbohydrate (Jones, 1978)

(2) Based on the classification scheme proposed by Schleifer and Kandler (1972)

(3) Most strains give a negative reaction, atypical strains show growth (Chazaud, 1978; Garvie, 1980, pers. comm.)

ND Data not available

Considerable progress has also been made in the areas of carbohydrate and protein metabolism and has been reviewed (Lawrence *et al.*, 1976; Lawrence and Thomas, 1979).

The cultures of lactic streptococci which are used in the manufacture of cheese and other fermented dairy products are called starters. The three main types of starter used commercially have been classified by Lawrence *et al.* (1976) as follows:

- A. Single-strain starters: single strains of *Str. cremoris* and less commonly *Str. lactis*. These are used in pairs in some factories in New Zealand and in Scotland but also singly in Australia.
- B. Multi-strain starters: defined mixtures of three or more single strains of *Str. cremoris* and/or *Str. lactis*. *Leuconstoc* and *Str. diacetylactis* strains may also be used. Multiple-strain starters are frequently referred to as mixed-strain starters in the United States of America.
- C. Mixed-strain starters: mixtures of strains of *Str. cremoris*, *Str. lactis*, *Str. diacetylactis* and *leuconstocs*. The identity of the component strains is frequently unknown to the user and their composition may vary on subculture.

Starters play a vital part in the manufacture of cheese. They produce the lactic acid which influences important quality characteristics such as texture, taste, moisture content, and freedom from pathogenic micro-organisms and their toxins in the mature cheese. The rate of acid production is critical in the manufacture of certain varieties, e.g. Cheddar. Starters are also required to produce acid at a reproducible rate from day to day especially in mechanised cheese production units. The negative redox potential created by starter growth in cheese may also aid in preservation and the development of flavour (Law, Castanon

and Sharpe, 1976). Additionally antibiotic substances produced by starters, e.g. nisin (Babel, 1977) may also have a role in preservation.

The failure of starters to produce sufficient acid at any stage in the cheese-making process can result in sub-standard quality cheese and a disruption of the timed operation. Since cheese factories process large volumes of milk daily (many factories in the United Kingdom process in excess of 100 thousand gallons a day at the peak of milk production) persistent starter problems could involve cheese factories in substantial financial losses.

Infection of starter cultures with a bacterial virus called a bacteriophage is virtually universally recognised as the major single cause of slow acid production and/or fermentation failure by this industrially significant bacterial group (Elliker, 1950; Whitehead, 1953; Christensen, 1972; Crawford, 1972; Lawrence *et al.*, 1976; Jespersen, 1977; Czulak *et al.*, 1979; Lawrence and Thomas, 1979; Walker, Mullan and Muir, 1981). The effects of bacteriophage infection are perhaps most evident in the manufacture of Cheddar cheese where rapid growth of lactic streptococci, sufficient to produce the equivalent of about 0.75% lactic acid, is required within a period of about 5 h. But phage is also capable of causing difficulties in the manufacture of other cheese varieties, ripened cream for butter manufacture and in the production of various fermented milks (Whitehead, 1953; Collins, 1962; Stadhouders, 1976; Lawrence, 1978; Saxelin *et al.*, 1979).

Infection with bacteriophage during cheesemaking can create a number of problems. The magnitude of the infection and the degree of replication of the bacteriophage will determine the extent of starter cell lysis and whether acid production is slowed or stopped. In addition to the disruption of the timed operations, production of insufficient lactic acid can lead to



financial loss, an inferior or worthless product or favourable conditions for the growth of pathogens, spoilage bacteria or toxin production (Elliker, 1950).

#### LACTIC STREPTOCOCCAL BACTERIOPHAGES

The infection of a growing bacterial culture with phage is initiated by the adsorption of the phage to the host cell. The specificity of adsorption of lactic streptococcal phages to lactic streptococci and the location of phage receptor substances have been studied (Oram and Reiter, 1968; Oram, 1971) and has been reviewed (Reiter, 1973; Lawrence *et al.*, 1976).

Lactic streptococci have been shown to have different receptor sites that may reside in both the cell wall and plasma membrane. Some phages may be relatively strain specific attacking only one or two strains but others show less specificity and may even attack strains of different species, i.e. a phage may attack strains of *Str. lactis*, *Str. cremoris* and *Str. diacetylactis* (Henning *et al.*, 1968; Nyiendo, 1974; Chopin, Chopin and Roux, 1976; Lawrence *et al.*, 1976). Although many strains are resistant to a specific phage because the phage cannot adsorb to receptor sites some strains which allow adsorption are also phage resistant (Oram and Reiter, 1968). The resistance of the latter has been attributed to lysogenic immunity or to the operation of a modification/restriction (M/R) system (Lawrence *et al.*, 1976).

Inorganic cations are required for adsorption (Cherry and Watson, 1949). However, calcium which is required for phage multiplication (Shew, 1949; Potter and Nelson, 1952) is not a specific requirement since monovalent cations are apparently just as effective (Reiter and Møller-Madsen, 1963).

The methods whereby phages eject or introduce their nucleic acid into lactic streptococci remain to be determined. An injection mechanism

is unlikely since the vast majority of lactic phages do not possess a contractile tail (Lawrence *et al.*, 1976). However a role for calcium and related ions in the process of DNA introduction has been postulated (Potter and Nelson, 1953). Following the introduction of phage DNA into a susceptible host, cell lysis and the release of newly formed viral particles occurs. In addition to phage, some phage-host systems also produce significant concentrations of a phage-induced lytic enzyme called phage lysin or lysozyme (Reiter and Oram, 1963; Oram and Reiter, 1965; Tourville and Johnstone, 1966). Unlike phage which is relatively strain specific, lysin is apparently unspecific, lysing strains on which the phage does not normally multiply (Oram and Reiter, 1965). Latent periods and burst sizes of some lactic streptococcal phages have been reported by several workers as a function of growth in different media and at different temperatures (Lawrence *et al.*, 1976).

Lysogenic or temperate phages also exist. Unlike the action of a lytic phage discussed previously, temperate phages do not normally kill their hosts. Instead the phage enters into a lysogenic relationship with its host in which the phage genome or a replica of it, becomes inserted into the bacterial chromosome. This relationship is very stable and is transmitted to following generations of the host.

Lysis of many strains of lactic streptococci has been reported after treatment with mitomycin C or ultraviolet light (McKay and Baldwin, 1973; Kozak *et al.*, 1973; Lowrie, 1974; Huggins and Sandine, 1977). Because the presence of phage in lysates was shown by electron microscopy and less frequently by the isolation of a sensitive indicator strain it was assumed that strains which exhibited lysis were lysogenic.

However, the first formal proof of lysogeny in this group was elegantly demonstrated by Gasson and Davies (1980). These workers isolated a non-lysogenic clone from a culture which lysed upon exposure to UV light or mitomycin C. The lysogenic state in the parent strain was proven by infecting the 'cured' strain with the temperate phage and re-establishing lysogeny.

Although the origin of phage active against streptococci is unknown, lysogenic starter culture strains have been suspected for some time as a possible reservoir (Reiter, 1949; Czulak and Naylor, 1956). There is now an increasing volume of circumstantial evidence (reviewed by Lawrence *et al.*, 1978) that the main source of phage in cheese factories is from the starter cultures themselves. However, lysogenic strains of *Str. lactis* present in the raw milk must also be considered as a possible source (Heap, Limsowtin and Lawrence, 1978; Lawrence *et al.*, 1978).

Despite several reports indicating the necessity for an acceptable nomenclature system for lactic streptococcal phages (Zehren and Whitehead, 1954; Keogh, 1973; Nyiendo, 1974; Mullan, 1979) no such system has yet been developed. Phages are still generally named after the initial host (Mullan, 1979). The problem is further complicated by the absence of a satisfactory classification system for the host strain (Crawford, 1972; Davies *et al.*, 1981) and by the discovery of temperate or lysogenic phages (Lawrence *et al.*, 1976). The classification of streptococcal phages has been reviewed (Lawrence *et al.*, 1976; Mullan, 1977).

All Group N phages analysed to date have contained only double stranded DNA with guanine-cytosine contents ranging from 32.7 to 40.7% (Henning, Sandine and Elliker, 1968; Nyiendo, 1974).

The majority of lactic streptococcal phages isolated can be classified within group B of Bradley's (Bradley, 1967) morphological classification system (Nyiendo, 1974; Lawrence *et al.*, 1976; Tsaneva, 1976; Huggins and Sandine, 1977; Heap, Limsowtin and Lawrence, 1978; Lawrence, 1978; Lawrence, Heap and Limsowtin, 1978; Sozzi *et al.*, 1978). Only one phage which could be classified within group C has been isolated (Saxelin *et al.*, 1979). Keogh and Shimmin (1974) reported a phage for a *Str. lactis* strain (C10) which apparently possessed a contractile tail. If this is so, this phage could be classified within Bradley's group A. Both prolate polyhedral and isometric head shapes have been found.

The group B phages have been further differentiated on the basis of head size and shape into small isometric, large isometric and prolate (Lawrence, 1978). The small isometric phages can be further subdivided into three groups on the basis of tail length and the presence or absence of a collar giving a total of five morphological groups. Apparently no further morphological groupings are justified at present (Lawrence, 1978).

Viruses active against lactic streptococci are generally enumerated using the double agar plaque assay as described by Adams (1959) using suitable complex media. The factors which influence plaque formation by lactic streptococcal phages include choice of growth medium, pH, temperature at which adsorption occurs, the ionic environment, incubation temperature, diluent composition and the choice of indicator strain (Mullan, 1979).

## BACTERIOPHAGE CONTROL IN CHEESE MANUFACTURE

Measures used to control phage in cheesemaking include aseptic techniques and special equipment for starter propagation (Whitehead and Hunter, 1945; Jones, 1956; Lewis, 1956; Ashton, 1963), phage inhibitory media (Babel, 1957; Hargrove, McDonough and Titlsler, 1961; Gulstrom *et al.*, 1979), the elimination or reduction of the ripening period used in the manufacture of certain cheeses (Hunter, 1944; Meanwell and Thompson, 1959; Keogh, 1973), aerosol disinfection of the air in the starter room and/or cheese production areas (Wolf, Nichols and Ineson, 1946; Walker *et al.*, 1981), the use of air filtration systems in which the air in the factory is exchanged with sterile air several times an hour (Thibodeau and Rendak, 1962), special cleaning and sterilising routines (Lawrence and Pearce, 1972; Hynd, 1976), the use of culture rotations (Anderson and Meanwell, 1942; Whitehead and Hunter, 1947), and the use of cultures composed of two or more phage-unrelated strains (Whitehead and Hunter, 1947; Collins, 1952b; Czulak and Naylor, 1956; Robertson, 1966b; Lawrence and Pearce, 1968; Breen, 1969; Lawrence and Pearce, 1972; Limsowtin, Heap and Lawrence, 1977; Daniell, Sandine and Burningham, 1979). Because this area has been extensively reviewed (Whitehead, 1953; Czulak and Naylor, 1956; Babel, 1962; Collins, 1962; Reiter and Møller-Madsen, 1963; Robertson, 1966a; Crawford, 1972; Lawrence and Pearce, 1972; Reiter, 1973; Lawrence *et al.*, 1976; Hull, 1977; Mullan, 1977; Sandine, 1977; Lawrence, 1978; Lawrence *et al.*, 1978), including one by the author (Mullan, 1977), further discussion will be limited to the use of cultures composed of phage-unrelated strains.

The use of cultures containing two or more phage-unrelated strains has played an important part in phage control schemes in cheese factories. Many phages have a relatively narrow host range and exhibit sufficient strain specificity to enable the use of cultures composed of phage-unrelated strains as part of a phage control programme.

The advantages of having a starter composed of a number of different strains is that phage infection will not normally markedly affect acid production because a strain lysed by phage would be replaced by the growth of another phage-unrelated or resistant strain (Collins, 1955).

The earliest cultures used which contained two or more phage-unrelated strains were commercial mixed-strain cultures which are still used widely today. However, it is difficult to maintain a constant mixed-strain starter as one strain may dominate after a few transfers. If strain domination occurs then phage infection can result in total cessation of acid production. Although mixed-strain starters have been reported as rarely showing complete cessation of acid production when infected with phage (Reiter and Møller-Madsen, 1963), retardation of acid production has been reported (Nelson, Harriman and Hammer, 1939; Anderson and Meanwell, 1942; Johns, 1943). In addition to retarded acid production, inhibition of acid production as drastic and sudden as that usually encountered with single-strain starters has also been experienced with mixed-strain starters (Johns and Katznelson, 1941; Babel, 1946; Collins, 1952a; Mullan and Walker, 1979, unpublished information).

The use of single strains of lactic streptococci for Cheddar <sup>and Cox</sup> cheesemaking was developed in New Zealand by Whitehead (1934). Single-strain starters were introduced to overcome textural problems in the cheese (the leuconstoc and *Str. diacetylactis* strains in the mixed-strain

cultures produced gas which gave open textured cheese) and to obtain a more uniform rate of acid production from day to day. This uniform rate of acid production, which is difficult to obtain with mixed-strain cultures (Lawrence and Pearce, 1972) made the use of such starters appear attractive to cheesemakers. However, the introduction of single strains created ideal conditions for the demonstration of phage action.

Subsequent to finding bacteriophage as the causal agent of starter failure New Zealand workers accumulated several phage-unrelated single strain cultures and introduced the use of paired-strain cultures, in a four-day rotation sequence (Whitehead and Hunter, 1947). Additional security was obtained by the use of a culture containing two phage-unrelated strains since in the advent of one strain being infected the remaining strain should continue to produce acid and enable cheese manufacture to continue (Whitehead and Hunter, 1947; Robertson, 1966b; Lawrence and Pearce, 1968). The use of this paired strain culture system combined with additional phage control measures (Lawrence and Pearce, 1972) worked relatively well up to the early 1970's.

The increase in milk processing capacity of many cheese factories in New Zealand in recent years has introduced new phage problems for some factories, particularly in factories in which the vats are refilled several times a day (multifill factories). The large number of starter strains necessary to construct a rotation for such multifill factories, often leads to cross phage relationships (Pearce *et al.*, 1970). Furthermore, where the first vats are run before the last vats are set, or in a double or multifill factory where a related starter is used in the second or third fills, sufficient phage will be produced by some starters to markedly slow or even stop acid production in later vats (Breen, 1969; Lawrence

*et al.*, 1976). Because of these difficulties the paired strain system which was formerly used in New Zealand for more than 40 years has been largely replaced with a multiple-strain starter containing a defined blend of phage-unrelated strains.

Strains for inclusion in multi-strain cultures are selected on the basis of physiological and phage sensitivity criteria - in all 14 test procedures are used (Lawrence *et al.*, 1978). The advantages of using multiple-strain cultures as described by the New Zealand workers include the possibility of using the same culture continuously for cheese manufacture, i.e. rotations are no longer required and that there is increased protection against phage-induced starter failure; the chance of all the component strains being infected simultaneously with their specific phages is unlikely. The multiple-starter system is apparently a success in New Zealand. One such culture has been used continuously for over three seasons to produce good quality cheese and approximately half of the cheese produced in New Zealand in 1979 was manufactured with bulk starter inoculated with a multiple-strain culture (Richardson *et al.*, 1980). While the successful use of multiple-strain cultures has been reported in the United States (Daniell *et al.*, 1979; Richardson *et al.*, 1980) failure of multiple-strain cultures has also been reported in the United States (Muskowitz, Staehler and La Belle, 1979) and in Scotland (Hynd, 1980, pers. comm.). The reasons for these failures were not determined.



## PROPOSED INVESTIGATION, GENERAL CONSIDERATIONS AND OBJECTIVES

Despite extensive research since the discovery of phage by Whitehead and Cox (1935) phage remains the major single cause of slow acid production in cheese manufacture.

The use of cultures containing two or more phage-unrelated strains has played a major part in phage control schemes. The advantage of having a starter composed of a number of different strains is that infection of one of the component strains with its specific phage, will not normally markedly affect acid production because the surviving strain or strains will continue to produce acid and enable cheese manufacture to continue.

The work contained in this thesis relates to a previously undocumented phage for *Str. lactis* C2, designated  $\phi$ C2(W), which when added to paired and multi-strain cultures containing C2 and other strains of lactic streptococci, normally resistant to this phage, inhibits acid production. This study had several aims:

- (1) To characterise the  $\phi$ C2(W) phage and to investigate the activity of the phage towards cultures of lactic streptococci. This work is reported in Chapter 1.
- (2) As a result of the above study evidence was obtained that the  $\phi$ C2(W)-C2 phage-host system produced a lytic agent which lysed phage sensitive and insensitive strains. Accordingly an attempt was made to isolate, purify and characterise the lytic agent. This work is reported in Chapter 2.
- (3) As a result of the work undertaken in Chapters 1 and 2 it was established that  $\phi$ C2(W) induces *Str. lactis* C2 to produce an enzyme which lyses the cell walls of Group N streptococci. Because of the

current interest in the genetics of lactic streptococci and the requirement for a gentle and effective method for cell wall removal a preliminary study of factors which affect the sensitivity of lactic streptococci to lysis by  $\phi C2(W)$ -induced lysin was undertaken. This work is reported in Chapter 3.

(4) Phage-induced lysins have been implicated in the processes involved in the entry of phage DNA into the bacterial cytoplasm (Reiter, 1973). This worker has suggested that lysin-resistant mutants should be phage resistant. Attempts to isolate and study lysin-resistant lactic streptococci are reported in Chapter 4.

It was envisaged that the knowledge gained from such a study would provide a deeper insight into the action of lactic streptococcal phages on lactic streptococci and by establishing the mechanisms involved in the inhibition of cultures by  $\phi C2(W)$ , measures might be developed to control the inhibitory phenomenon.

## CHAPTER I

CHARACTERISATION OF THE  $\phi$ C2(W)-C2 PHAGE-HOST SYSTEM

AND ATTEMPTS TO ELUCIDATE THE MECHANISM

OF THE NASCENT PHAGE PHENOMENON

## INTRODUCTION

Evans (1934) first used the term nascent phage phenomenon to describe the inhibition of growth observed when a culture containing two phage-unrelated strains demonstrated growth inhibition when infected with a phage specific for one of the strains. The term nascent was used to imply a special potency for phage at the instant of release from the host which 'normal' phage did not possess.

A similar phenomenon has been reported for lactic streptococci (Anderson and Meanwell, 1942; Hunter, 1943; Nichols and Wolf, 1945) in which paired or multiple-strain cultures (containing phage-unrelated strains) exhibited slow acid production when infected with a phage specific for one of the component strains. The nature of the inhibition has been studied by several workers. Collins (1952a) found that two out of 14 phages studied were capable of inhibiting growth and acid production of several strains which were normally resistant to both viruses. This worker questioned the use of the term nascent since he showed that the presence of the propagating host was not essential for demonstration of the phenomenon and went on to show that high titre phage lysates (free of propagating host) could cause inhibition. Collins attempted to determine whether the inhibition observed with phage lysates was due to phage *per se* or to another inhibitor. Since both phage and the ability of the lysate to inhibit the growth of strains normally resistant to the phage (non-homologous strains) were lost after steaming for 3-4 min, it was concluded that phage *per se* was involved in the inhibitory process. A similar study was undertaken by Whitehead, Hunter and Cox (1952). They also established that the presence of the propagating host was not necessary and that inhibition of normally phage-resistant strains could be obtained by using high titre phage preparations. In experiments designed to determine

whether inhibition was due to phage or to an inhibitory substance present in phage lysates the following results were obtained:

- (i) both phage and the inhibitory activity of lysates were destroyed by a heat treatment of 66°C for 5 min at pH 5.
- (ii) the inhibitory activity of lysates was associated with the phage and was independent of the propagating host.
- (iii) strains which absorbed high levels of phage were inhibited to a larger extent than strains which exhibited low adsorption.
- (iv) microscopic observation of infected cultures was consistent with a drastic interference with bacterial metabolism.

From these results Whitehead *et al.* (1952) concluded that the nascent phage phenomenon was due to a host-phage interaction which disrupted normal host metabolism and inhibited growth and acid production without phage production. These workers also obtained data which did not agree with this hypothesis. Anomalous results were obtained with three strains for which a relationship between the level of phage adsorption and sensitivity to the lytic agent in phage lysates was not established e.g. a strain which showed high adsorption was virtually resistant to inhibition, the converse was also found. Further evidence for the direct role of phage *per se* in the nascent process was presented by Whitehead, East and McIntosh (1953).

An alternative explanation for nascent effects in which phage is only indirectly involved is also possible. Bronfenbrenner and Muckenfuss (1927) recognised that phage lysates contain a lytic agent other than phage. This agent, a 'ferment like' substance, differed from phage because it lysed only dead staphylococci. It was absorbed to clay filters,

inactivated on storage at ambient temperature, more heat labile than phage and did not diffuse through collodion membranes. This description fits the staphylococcal virolysin (or phage lysin) described much later by Ralston *et al.* (1955). This phage-induced enzyme lysed dead cells, although living cells were lysed if the homologous phage was present. Wollman and Wollman (1933), cited by Tsugita (1971), regarded the lysis of a phage-resistant strain grown in a mixture containing a propagating host as due only indirectly to the phage, which they thought released specific "diastases" in addition to phage from lysing host cells. These diastases caused the lysis of the phage-resistant strain. The discovery of hen egg lysozyme by Meyer *et al.* (1936) was helpful in clarifying the role of phage-induced lysins in the nascent process. Pirie (1939) suggested that an enzyme (not of bacterial origin) is released upon lysis of bacteria by phages and it acts on the bacterial cell wall and increases the content of reducing sugar in the growth medium in a similar way to lysozyme.

Pette (1953) first demonstrated lysin production by lactic streptococcal phages and showed that lysin was produced by phages which exhibited secondary zones of lysis around plaques (haloes). Halo formation is widely regarded as a characteristic of a lysin-producing phage (Tsugita, 1971). Partially purified lysin, lysed strains which were resistant to the lysin producing phage.

Further evidence for the role of phage lysins in the nascent phage phenomenon is apparent from work by Naylor and Czulak (1956). These workers found that if *Str. lactis* C10 and *Str. cremoris* C1 were grown together, and infected with certain phages for C10, ( $\phi$ C10-1,  $\phi$ C10-11 but not  $\phi$ C10-111) C1 was lysed. *Str. cremoris* C1 did not propagate or adsorb the phages for C10. Further study revealed that lysates of  $\phi$ C10-1 and  $\phi$ C10-11 contained a lytic agent other than phage. The lytic agent could be

differentiated from phage by heat sensitivity; a treatment of 60°C for 5 min inactivated the lytic activity but not the phage. Fractionation of the two activities, was also obtained by Seitz filtration whereby lytic activity but not phage was lost. In agreement with the data of Pette (1953) the two lysin producing phages both exhibited halo formation whereas  $\phi$ C10-111 gave plaques which were not surrounded by a halo.

More recently, further evidence for the role of phage-induced lysins in the nascent phage phenomenon has been obtained from the work of Oram and Reiter (1965), Tourville and Johnstone (1966) and Tourville and Tokuda (1967). These workers isolated and partially characterised several phage lysins and showed that the lysins were phage induced and, unlike phage, strain unspecific. In addition Oram and Reiter (1965) demonstrated the lysis - from - without of several streptococci with high multiplicities of phage (13.5) due to the action of phage tail lysin.

In this section the  $\phi$ C2(W)-C2 system is partially characterised, the nascent effect of  $\phi$ C2(W) is demonstrated, and attempts are made to determine the mechanism of the nascent phenomenon.

## MATERIALS AND METHODS

### ORIGIN OF CULTURES AND BACTERIOPHAGES

*Str. lactis* strains C2, C10, ML3, 712 and *Str. cremoris* E8 were obtained from the culture collection maintained at this Department. Bacteriophages designated  $\phi$ C2(W),  $\phi$ P2 and  $\phi$ E8 were also obtained from this source.  $\phi$ C2(W) was originally isolated by Mr. A. L. Walker, from an infected culture, in 1969 and had been stored in broth at 5°C for about eight years when reactivated by the author. *Str. lactis* C6 and *Str. diacetylactis* DRC2 were obtained from Dr. B. Keogh, Commonwealth Scientific and Industrial Research Organisation, Melbourne, Australia. Phages  $\phi$ DRC2 and  $\phi$ ML1 were also obtained from Dr. Keogh. *Str. lactis* ML8 and *Str. cremoris* strains US3, AM1 and US3 in addition to phages  $\phi$ C10 and  $\phi$ ML8(1) were obtained from Dr. T. Cogan, The Agricultural Institute, Fermoy, County Cork, Ireland. *Str. cremoris* SC1 and  $\phi$ SC1 were obtained from Dr. W. E. Sandine, Oregon State University, USA. *Str. cremoris* strains C13 and SK11 and  $\phi$ ML3 were obtained from Dr. Garvie, National Collection of Dairy Organisms, N.I.R.D., Shinfield, Berks., England.  $\phi$ 712 was obtained from Dr. M. Gasson, also of the same Institute.

### CULTURE MAINTENANCE

Working cultures were maintained by weekly transfer of a 0.1% (v/v) inoculum in sterile reconstituted skim-milk and incubated at 30°C for 14-18 h. Skim-milk was reconstituted using 10% non-fat-milk solids and sterilised by autoclaving at 115°C for 15 min. When cultures were required for studies in milk or broth media they were subcultured twice in the appropriate medium prior to use. Between transfers, cultures were stored at 4-5°C. For longer term storage, 3 ml of sterile reconstituted skim-milk (RSM) were inoculated with 1% (v/v) culture and frozen at -30°C.



Periodically, working cultures were streaked onto one of the agar media cited in the text and culture morphology and catalase reaction determined to ensure culture purity.

All cultures were tested using the streak technique of Mullan and Walker (1979) to confirm species identity. In addition the ability of *Str. lactis* C2 to ferment certain carbohydrates, grow in media containing 2, 4 and 6.5% (w/v) sodium chloride, grow at 22°, 30°, 38°, 40° and 45°C was determined. The sensitivity of this strain to certain phages was also tested. Some characteristics of the strain used in this study are shown in Table 1.1.

#### CARBOHYDRATE UTILISATION

Carbohydrate utilisation was studied in a medium essentially similar to M17 (Terzaghi and Sandine, 1975) except that the glycerophosphate component was omitted and other carbohydrate or related compounds were substituted for lactose. The basal medium contained the following ingredients dissolved in 1 l of distilled water: polypeptone (BBL), 5 g; phytone peptone (BBL), 5 g; lab lemco (Oxoid), 5 g; bromocresol purple (0.1% w/v aqueous solution), 2.0 ml. The medium was dispensed in 2.5 ml quantities and sterilised at 121°C for 15 min. Prior to carbohydrate utilisation studies, 2.5 ml of a sterile 1% (w/v) aqueous solution of carbohydrate were added to the basal medium; solutions were sterilised at 121°C for 15 min. The complete medium was inoculated with 0.1 ml of a late stationary phase culture (1% inoculum, 30°C, 16 h) and incubated at 30°C. Cultures were examined for acid production at intervals over a 72 h period. Acid production was denoted by the pH indicator changing from purple to yellow. None of the strains tested showed evidence of growth when inoculated into basal media which had not been supplemented by additional carbohydrate.

Table 1.1

Some characteristics of *Str. lactis* C2

<u>Acid from</u>		<u>Growth in PLGYG at</u>	
glucose	+	22°C	+
lactose	+	30°C	+
galactose	+	40°C	+
fructose	+	45°C	-
maltose	+		
dextrin	+		
mannitol	+	<u>Growth in PLGYG containing</u>	
sucrose	-	2% sodium chloride	+
gluconate	+	4% sodium chloride	+
$\beta$ -disodium glycerophosphate	-	6.5% sodium chloride	-

Sensitivity to phage

$\phi$ 712	+
$\phi$ ML3	+
$\phi$ C2(W)	+
$\phi$ ML8(1)	-
$\phi$ C10	-
$\phi$ DRC2	-
$\phi$ SC1	-
$\phi$ P2	-
$\phi$ ML1	-
$\phi$ E8	-

These characteristics were determined using methods described in text.

+ indicates a positive reaction; - indicates a negative reaction.

## PHAGE ASSAY

Phage plaque counts were determined by the soft agar overlay method (Adams, 1959; Mullan, 1979) on M17 agar (Terzaghi and Sandine, 1975) or on PLGYG agar (Mullan, 1977; Mullan, Daly and Fox, 1981b). The procedure followed has been described in detail (Mullan, 1977).

## HOST RANGE OF $\phi$ C2(W)

Loopfuls of phage preparations, diluted to give about  $10^7$  plaque-forming units/ml (pfu/ml), were spotted onto PLGYG double agar plates in which the soft agar had been inoculated with about  $10^8$  colony-forming units (cfu) of the strain under study (Mullan, 1977; Mullan *et al.*, 1981a). Plates were examined after 18 h incubation at 30°C for lytic zones.

## PREPARATION OF PHAGE STOCKS

PLGYG broth was supplemented with 5 mM  $\text{Ca}^{++}$ , inoculated with 1% culture and sufficient phage was added to give a concentration of about  $10^6$ - $10^7$  pfu/ml. Infected cultures were incubated at 30°C and lysed before 4 h. At 6 h, lysates were filter sterilised using a syringe mounted swinnex filtration unit (Millipore (U.K.) Ltd., Millipore House, Abbey Road, London) fitted with a 0.45  $\mu\text{m}$  membrane and 2 ml quantities dispensed aseptically into sterile bottles and frozen at -22°C. Phage suspensions prepared by this method contained  $10^9$ - $10^{10}$  pfu/ml and, as far as the phage in this study were concerned, were stable to freezing and thawing.

## ULTRASTRUCTURE OF $\phi$ C2(W)

Several procedures were used to produce high titre phage preparations for investigation by electron microscopy. Two methods yielded preparations of  $\phi$ C2(W) which were easily visible. In the first procedure, a PLGYG

broth lysate, containing about  $10^{10}$  pfu/ml, was concentrated to give about  $10^{13}$  pfu/ml by ultrafiltration. Concentration and partial purification of phage were achieved by the use of an Amicon Model 202 ultrafiltration cell (Amicon Corporation, Lexington, Mass. 02173, USA) fitted with a Nuclepore membrane (Nuclepore Corporation, Pleasanton, California 94566, USA), type C, of molecular weight cut off 100,000 daltons. The unit was pressured by nitrogen gas and was operated at 30 p.s.i. Ultrafiltration experiments were performed at 5°C unless stated otherwise. Partial purification of the phage-containing concentrate was achieved by washing the concentrate several times with quarter strength Ringer solution. The second method utilised the observation that during the isolation and purification of phage lysin (Chapter 2) both lysin and phage were bound and easily eluted from Amberlite CG50 ion exchange resins. Partially purified phage containing eluent from a CG50 column was concentrated and further purified by ultrafiltration to give about  $10^{13}$  pfu/ml as described above. Phage suspension (50  $\mu$ l) was negatively strained with an equal volume of a 3% (w/v) solution of phosphotungstic acid (pH 7.0). Ten  $\mu$ l of the mixture were placed in a copper grid coated with formvar and blotted dry. The grid was examined at an accelerating voltage of 80 kV using a Siemens Elmiskopf 101 electron microscope.

#### ASSAY OF LYTIC ACTIVITY

The activity of lytic enzymes can be determined by methods based on either turbidimetry or the estimation of some solubilised cell wall component. In this study turbidimetry was used. A standardised suspension of cells in buffer was mixed with a sample of lysin-containing material. The lysin caused the lysis of the cell suspension and a reduction in the optical density (O.D.). The enzyme activity was calculated from the decrease in O.D.

### Preparation of substrate

PLGYG broth (Mullan, 1977; Mullan *et al.*, 1981b) was inoculated with 1% culture and incubated at 30°C for 5 h. After incubation, cultures were cooled to 10°C or less and the cells pelleted by centrifugation at 4,000 r.p.m. for 10 min. If freshly harvested cells were used, the cells were washed twice in 0.1 M potassium phosphate buffer pH 6.8 (standard buffer). If freeze-dried cells were used, the cells were washed twice in distilled water and frozen in this medium. Cells were freeze-dried in 10-20 ml quantities over a 48 h period using an Edwards Freeze Drier (Model no. 10-F023-880), Manor Road, Crawley, West Sussex, England. Freeze dried cells were stored in the desiccated form at 5°C.

### Assay procedure

Cells (frozen and thawed, freshly harvested or freeze-dried) were suspended in standard phosphate buffer to give an O.D.<sub>450</sub> of 0.62-0.75 using a Spectronic 20 Spectrophotometer (Bausch and Lomb Inc., Rochester, N.Y., USA). Five ml of standardised cell suspension were added to a cuvette and tempered to 37° ± 0.1°C in a thermostatically controlled water bath and depending upon lytic activity of the sample, 0.1-1.0 ml of lysin-containing solution was added. The mixture was mixed briefly with a vortex mixer and O.D.<sub>450</sub> readings taken at 30 sec intervals over a 2-6 min period. Samples were mixed periodically. O.D. readings were plotted against time and only the values on the linear portion of the graphs were used for calculation. With some preparations e.g. *Str. lactis* C10, appreciable autolysis was observed on occasions and compensation for autolytic activity was necessary if low titre lysin preparations were assayed. Little or no autolytic activity was apparent with freeze-dried cells of C2 suspended in standard buffer for 30 min at 37°C. One unit of enzyme activity was

defined as that concentration of phage lysin which gave a decrease in absorbancy of 0.001 units/min under the reaction conditions defined above. Unless stated otherwise lysin assays were performed with freeze-dried cells of C2. In later experiments a Bausch and Lomb Model 1200 Clinical Analyser was used. This instrument was fitted with a printer and thermostatically controlled cell. In some experiments a Shimadzu UV 120-01 spectrophotometer (14-5, Uchikanda 1-chome, Chiyoda-ku, Tokyo, Japan), fitted with a thermoregulated cell holder and coupled to a chart recorder was used.

#### Relationship between optical density and colony forming units (cfu) for *Str. lactis* C2

To enable the reader to relate cfu and O.D.<sub>450</sub> a standard curve was prepared and is shown in Figure 1.1. The curve was obtained by harvesting a log phase culture of C2 by centrifugation. The pellet was washed once in standard phosphate buffer and the washed cells diluted with buffer to give a series of cell suspensions of known O.D.<sub>450</sub>. The number of cfu/ml was obtained by the pour plate method using PLGYG agar. Dilutions were made in quarter strength Ringer solution and plates were incubated at 30°C for 48 h. The curve obtained conformed with the equation  $y = 1.39x + 7.11$ . The correlation coefficient (linear R) was 0.998.

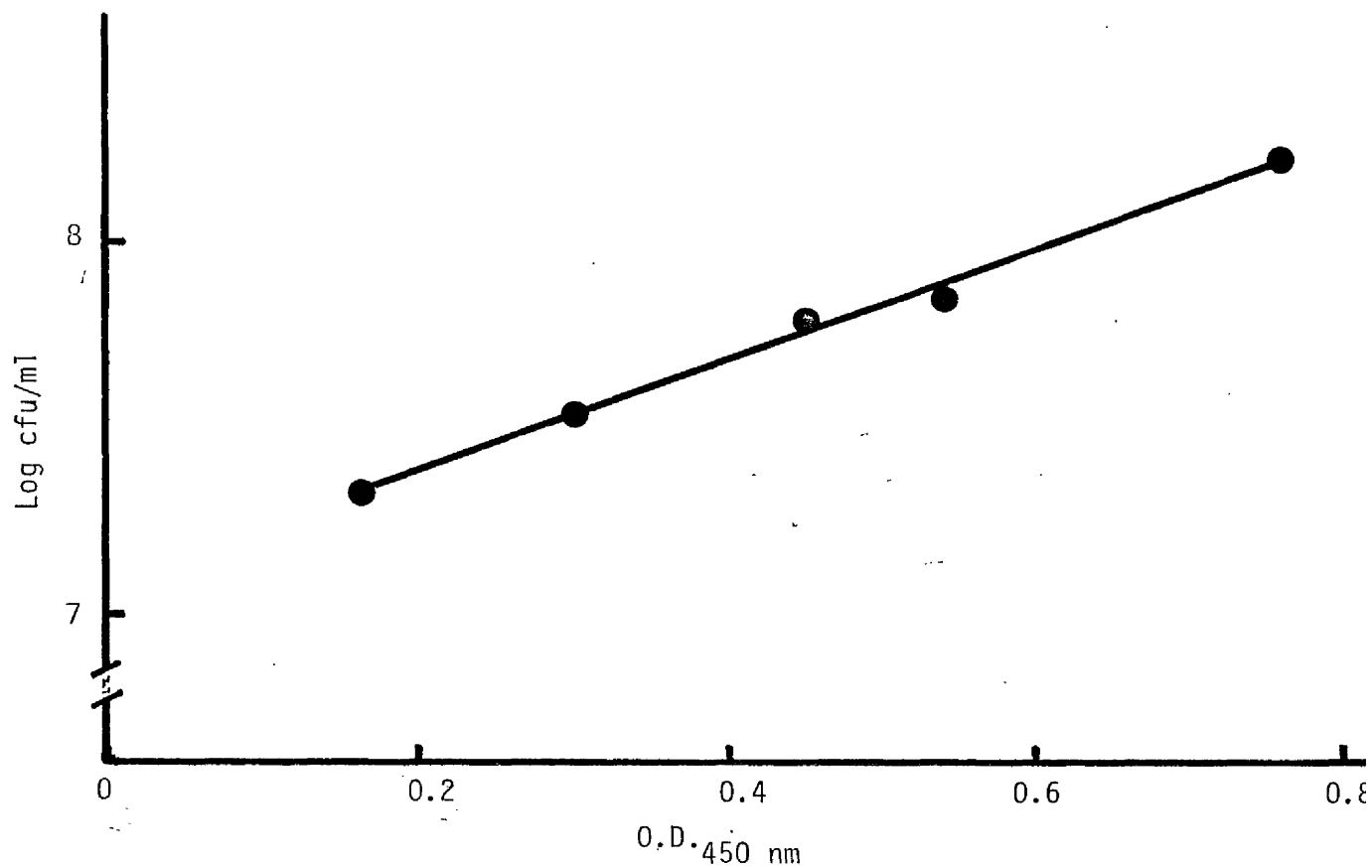
#### PHAGE REMOVAL FROM $\phi$ C2(W) LYSATES

In preliminary experiments two methods of phage removal were studied:- precipitation and centrifugation.

##### Precipitation

Acetone at -20°C was added to lysates, held in a water bath at 4°C, to give a concentration of 40% (v/v) (Oram and Reiter, 1965). A dense

Figure 1.1 Relationship between O.D.<sub>450</sub> and cfu/ml for  
*Str. lactis* C2



Log phase cells of *Str. lactis* were recovered from PLGYG broth by centrifugation and suspended in fresh medium to give a series of cell suspensions of different O.D. values. cfu were determined using the pour plate method and PLGYG agar. Results shown are means of two determinations

white precipitate formed immediately and was left to settle overnight at 5°C. Next morning the clear liquid was carefully decanted from the precipitate and filtered through Whatman No. 40 paper, using suction.

### Centrifugation

Phage was sedimented by centrifugation at 75,000 x g for 4 h in a MSE high speed centrifuge. Temperature was maintained at 4°C during centrifugation.

### ADSORPTION OF PHAGE TO LACTIC STREPTOCOCCI

The method used has been described in detail (Mullan, 1977; Mullan *et al.*, 1981a) and was similar to that described by Keogh (1973) except that PLGYG broth and agar media were used.

### LYTIC ACTIVITY IN THE SUPERNATANTS OF BROKEN AND UNBROKEN CELLS OF *Str. lactis* C2 INFECTED WITH $\phi$ C2(W)

PLGYG broth was inoculated with C2 and grown at 30°C to give an O.D.<sub>450</sub> of about 0.8. This corresponds to about  $2 \times 10^8$  cfu/ml. At this point 5 mM  $\text{Ca}^{++}$  and sufficient phage to give a MOI of about ten were added. After mixing, a series of 10 ml cultures was prepared and placed in McCartney bottles. Cultures were incubated at 30°C and at intervals removed, chilled rapidly to <5°C in a circulating ethylene glycol solution at 2°C and either broken by sonication to obtain an estimate of the intracellular concentration of lysin or filtered to give a cell-free supernatant which was used to determine the extracellular concentration of lysin.

Sonication was performed using a Rapidis Ultrasonic Disintegrator, Model 350G (Ultrasonics Ltd., Shipley, Yorks, England) fitted with a 9.5 mm diameter tip. The tip (chilled to about 3°-5°C) was inserted into



culture bottles and was operated at power setting 7 and tuning setting 1.5 for 4 min. During sonication samples were cooled by contact with a circulating ethylene glycol solution at 2°C. The sample temperature did not exceed 9°C during cellular disruption. Sonication reduced the absorbance value to about 0.23. Broken cells were removed using a syringe mounted filtration unit containing a 0.45 µm filter and the filtrate was analysed for lysin. Sonication of filter sterilised broth lysates (containing about 100 u/ml of lysin) revealed that the sonication conditions described above had no significant effect on lytic activity.

#### DETERMINATION OF PROTEIN

Protein analyses were performed using the Bio-Rad protein assay (Bio-Rad Laboratory Ltd., 27 Homesdale Road, Bromley, Kent) according to the instructions given in Bio-Rad Technical Bulletin 1069. The Bio-Rad assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding of protein occurs and is a modification of the method described by Bradford (1976). Bovine serum albumin was used as the protein standard.

#### PREPARATION OF LYSIN-FREE PHAGE PREPARATIONS BY NEGATIVE CHROMATOGRAPHY ON THE DYE LIGAND GREEN A

In an attempt to purify  $\phi$ C2(W)-induced lysin by dye-ligand chromatography (Chapter 2) it was observed that phage lysin but not phage was bound by the ligand Green A. This observation formed the basis of the method described in this section for the preparation of high-titre preparations of  $\phi$ C2(W) which did not contain lysin.

### Small scale lysin removal

The Green A column was obtained from the Amicon Dyematrix Screening kit for protein purification (Amicon Ltd., Amicon House, 2 Kingsway, Woking, Surrey, UK) and contained 2 ml of gel. The gel was regenerated with 12 ml of a 0.5 M solution of sodium hydroxide, which also contained 6 M urea, to remove free dye. The column was equilibrated with 12 ml of 20 mM Tris (2-amino-2hydroxymethyl-1-3 propandiol hydrochloride), pH 6.8 as described in Amicon Technical publication 1-190 which accompanied the Dyematrix kit. Two ml of lysin-containing solution were applied to the column. The first ml of effluent was discarded. Ten ml of equilibration buffer were then added and the first 2 ml collected. This fraction contained phage but not lysin. Larger quantities of lysin-free phage preparations were obtained by chromatography on a larger column. A 30 x 1.5 cm column was packed with the Green A (Amicon Ltd., Amicon House, 2 Kingsway, Woking, Surrey, UK) which was suspended in equilibration buffer. The bed height was adjusted to 23 cm and free dye removed by elution with the regeneration solution. The column was washed free of regeneration solution and equilibrated with buffer until the pH reached 6.8. The void volume of the column was about 40 ml. Preliminary experiments established that the column had sufficient capacity to bind the lysin contained in a 100 ml volume of a crude PLGYG broth lysate or in 90 ml of partially purified lysin obtained by ion exchange on CG50 (Chapter 2). Lysin-containing material was applied to the column using a flow rate of  $60 \text{ ml h}^{-1}$  (linear flow rate of about  $34 \text{ ml cm h}^{-1}$ ). Matrex gel media have been reported to be run at flow rate up to 80 cm/h with only minimal compression (Amicon publication 1-191: Operating instructions for Matrex Gels Blue A, Red A, Orange A, Green A and Blue B). The void volume was discarded and the eluent collected. The equilibration buffer was used to displace the material

held up in the column. The lysin-free eluant was concentrated by ultrafiltration as described previously.

#### EFFECT OF INFECTING PAIRED PHAGE-UNRELATED SINGLE-STRAIN STARTER CULTURES WITH $\phi C2(W)$

Single-strain cultures were inoculated at the 1% (v/v) level into sterile reconstituted skim-milk (RSM) of 10% T.S. and incubated at 22°C for 16 h. Paired phage-unrelated cultures were blended to give mixtures containing similar cell populations by mixing 5 ml quantities of freshly coagulated cultures. The mixtures were used to inoculate sterile RSM at the 2% (v/v) level and divided into two portions, one of which was infected with sufficient  $\phi C2(W)$  to give  $1.4 \times 10^4$  pfu/ml. Phage-infected and control cultures were dispensed in 10 ml quantities into McCartney bottles and incubated at 30°C for 5 h. At this time the pH of both infected and control cultures and the phage titre in infected cultures were determined. For the latter 1 ml of 5% (v/v) lactic acid was added to precipitate the protein. The whey was titred after centrifugation at 5,000 x g for 10 min.

#### EFFECT OF INFECTING A MULTI-STRAIN CULTURE WITH $\phi C2(W)$

A multi-strain culture containing similar cell numbers of C2, C10 and ML8 was prepared essentially as described for the paired-strain mixtures and 4 ml of the starter added to 196 ml of sterile RSM. The RSM was then divided into three cultures of equal volume. Two of the cultures were infected with  $\phi C2(W)$  to give phage concentrations of about  $2 \times 10^2$  pfu/ml and  $2 \times 10^4$  pfu/ml respectively. The remaining culture served as the control. Control and infected cultures were dispensed in 5 ml quantities into sterile McCartney bottles, and incubated at 30°C. At intervals the bottles were removed and the acidity was measured by pH determination.

Phage replication was determined in infected cultures as previously described.

#### DETERMINATION OF THE MINIMUM CONTENT OF C2 REQUIRED FOR INHIBITION OF A MULTI-STRAIN CULTURE INFECTED WITH $\phi$ C2(W)

Five ml quantities of late stationary phase cultures (1%, 16 h, 22°C) of C10, ML8 and C2 in RSM were diluted with 5 ml of sterile uninoculated RSM and mixed with a vortex mixer. Dilution was necessary to allow accurate pipetting to enable the preparation of mixtures containing different levels of *Str. lactis* C2. The ML8 and C10 cultures were mixed and four 4 ml aliquots of the mixture were removed and placed in sterile test tubes to give a total of five tubes containing a mixture of C10 and ML8. Sufficient C2 culture was added to each tube to give multi-strain cultures containing 1, 5, 10, 20 and 50% (v/v) of C2 respectively. Duplicate 2 ml quantities of each mixture were removed and used to inoculate two 48 ml lots of RSM. One of the cultures served as a control and the remaining culture was infected with  $\phi$ C2(W) to give  $1.4 \times 10^4$  pfu/ml. A series of 5 ml cultures was prepared from each control and phage-infected culture and incubated at 30°C. At intervals cultures were examined for acid production.

#### REAGENTS

All reagents used were of 'Analar' or highest quality available and except where indicated in the text were obtained from BDH Chemicals, Poole, Dorset, England or Sigma, Sigma Chemicals, Fancy Road, Poole, Dorset.

## RESULTS

### PARTIAL CHARACTERISATION OF $\phi$ C2(W)

Classification of Group N streptococcal phages is still in the early stages of development. In the absence of an accepted classification scheme for these phages an attempt has been made to partially characterise  $\phi$ C2(W) by plaque morphology, host range, ultrastructure, latent period and burst size and the effect of temperature on phage replication. The object of this work was to differentiate the  $\phi$ C2(W) phages from other phages for C2 or the related strains 712 and ML3 (Davies *et al.*, 1981).

#### Plaque morphology

The  $\phi$ C2(W)-C2 phage-host system exhibits 'haloes' around plaques (Plate 1.1). Chloroform treatment (Tsugita, 1971) did not extend the zone of halo formation nor was it necessary to demonstrate the phenomenon. Slight halo formation was apparent after 18 h incubation at 30°C and became more pronounced with increasing incubation time. Halo formation was particularly pronounced after 48 h at 30°C. The phenomenon was accelerated if phage plates incubated at 30°C were removed after 6 h and transferred to 37°C for a further 14 h. This treatment gave plaques surrounded by haloes of size comparable with plaques held at 30°C for 48 h. Halo formation around plaques also occurred at 5°C. If phage plates were removed after 14-16 h incubation at 30°C, at which time only slight evidence of halo formation was apparent, and transferred to 5°C halo formation continued at a slow rate and was apparent after about two weeks incubation.  $\phi$ C2(W) produced plaques on PLGYG or M17 agar whether or not the medium was supplemented with  $\text{Ca}^{++}$ . Calcium addition, however, was required for halo formation and good zones of secondary lysis were obtained if M17 was supplemented with 10 mM  $\text{Ca}^{++}$ . Supplementation of assay media with  $\text{Ca}^{++}$

Plate 1.1      Morphology of plaques produced by  $\phi$ C2(W) on  
*Str. lactis* C2



Plaques were photographed against a dark background after 48 h incubation on M17 agar at 30°C

Table 1.2

Effect of  $\text{Ca}^{++}$  addition to M17 agar on the titre of  $\phi\text{C2(W)}$

Calcium addition (mM)	pfu/ml
0	$7.5 \times 10^{10}$
10	$13.0 \times 10^{10}$

increased the efficiency of plating, however (Table 1.2). Satisfactory demonstration of the halo phenomenon was also found to depend upon the buffering capacity of the phage assay medium. M17 agar consistently gave larger zones of secondary lysis than PLGYG agar but haloes of similar size were obtained if PLGYG was supplemented with sufficient glycerophosphate (GP) to give a medium containing a similar level of GP to M17.

#### Host range

$\phi$ C2(W) lysed *Str. lactis* strains C2, 712 and ML3. Strains DRC2, C10, ML8, SC1, E8, AM1, AM2, US3, SK11, R6, UG1246 and C6 did not propagate  $\phi$ C2(W).

#### Effect of temperature on the production of $\phi$ C2(W) by C2 in PLGYG broth

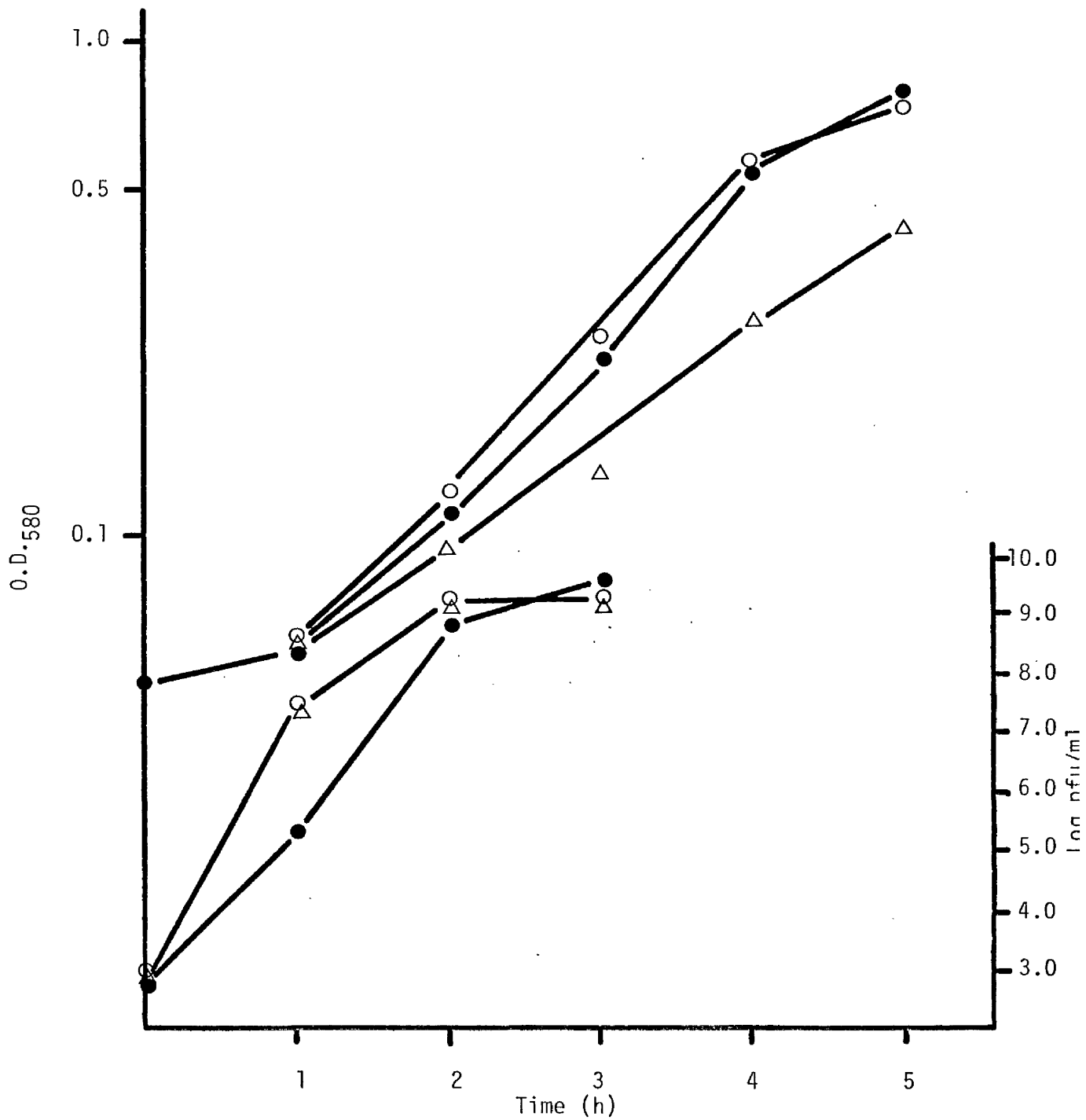
Temperatures in the region 30°-40°C have been reported to have a major effect on the replication of some Group N streptococcal phages (Mullan *et al.*, 1981a; Mullan *et al.*, 1981b). Mullan *et al.* (1981a) have suggested that the ability of streptococcal phage-host systems to propagate phage in the region 30°-40°C may be useful in the differentiation of some phages. It is apparent (Figure 1.2) that  $\phi$ C2(W) is not temperature-sensitive over the temperature range studied. For comparison the growth response of C2 at each temperature studied is also shown. It is obvious that the replication rate of  $\phi$ C2(W) is considerably greater than that of its host.

#### Ultrastructure of $\phi$ C2(W)

Electron microscopy (Plates 1.2a, 1.2b) revealed that  $\phi$ C2(W) had a prolate head and a non-contractile tail which did not appear to possess a terminal tail structure. The  $\phi$ C2(W) virus had a total particle length, head diameter, head length, and tail diameter of about 144-146 nm,

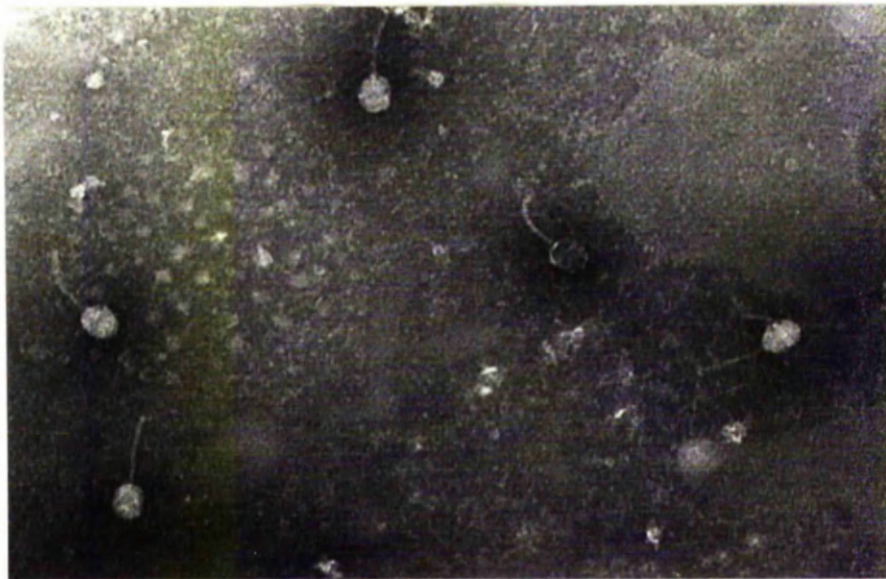


Figure 1.2 Effect of temperature on cell growth and  $\phi$ C2(W) production by *Str. lactis* C2 in PLGYG broth

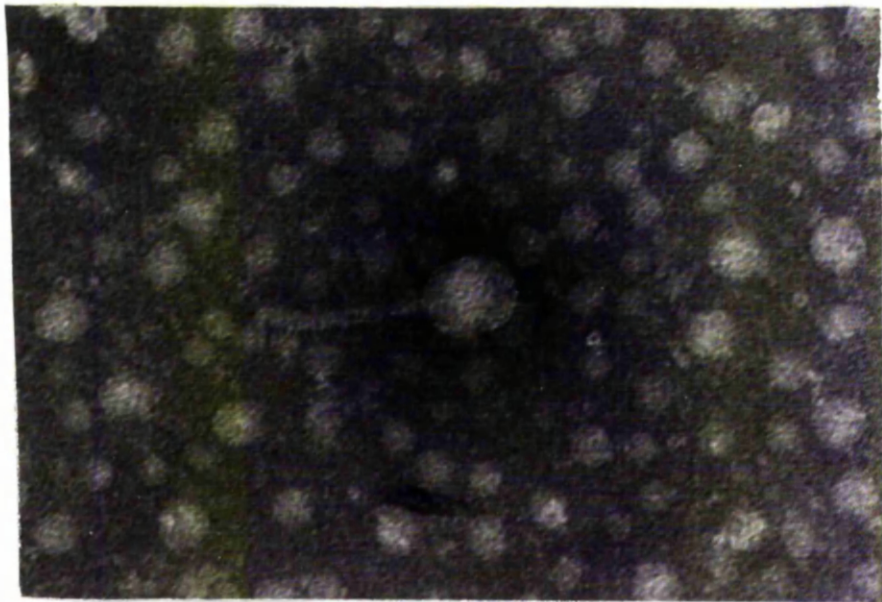


- , phage production and culture growth at 30°C, respectively
- , phage production and culture growth at 38°C, respectively
- △—, phage production and culture growth at 40°C, respectively

Plate 1.2 Particle morphology of  $\phi$ C2(W) negatively-stained with phosphotungstic acid



(a) x 100,000



(b) x 300,000

44-46 nm, 54-56 nm, 90 nm and 8.5 nm respectively. Phage ghosts are also apparent in Plate 1.2a.

#### Latent period and burst size of $\phi C2(W)$

The latent period, rise period and burst size of  $\phi C2(W)$  on C2 in PLGYG broth at 30°C was about 20 min, 35 min and 148 particles, respectively. The results of a typical experiment are shown in Figure 1.3.

#### EFFECT OF INFECTING PAIRED-STRAIN CULTURES WITH $\phi C2(W)$

Paired and multi-strain cultures containing phage-unrelated strains of Group N streptococci are widely used as starter cultures in the manufacture of cheese and other fermented dairy products (Lawrence *et al.*, 1976). The use of these cultures is widely accepted to give protection from fermentation failure resulting from phage infection, since in the event of phage infection the surviving strain or strains continue to produce acid and enable completion of the fermentation.

In this section experiments were designed to investigate the effect of infecting paired-strain cultures containing C2 with  $\phi C2(W)$ . The component strain present in each culture with C2 did not propagate  $\phi C2(W)$ .

Since inhibitory strains of Group N streptococci have been reported (for a review see Reiter and Møller-Madsen, 1963) preliminary experiments were undertaken to exclude any combinations which were inhibitory. Experiments performed using a streaking technique failed to reveal any inhibitory effects between C2 and the component strains of the paired strain cultures. The value of this technique in isolating streptococci which produce inhibitory substance against other strains was apparent from contributions presented at a recent International Circle of Dairy Research Leader's Conference by von Geiss and Teuber (1981). None of

Log pfu/ml

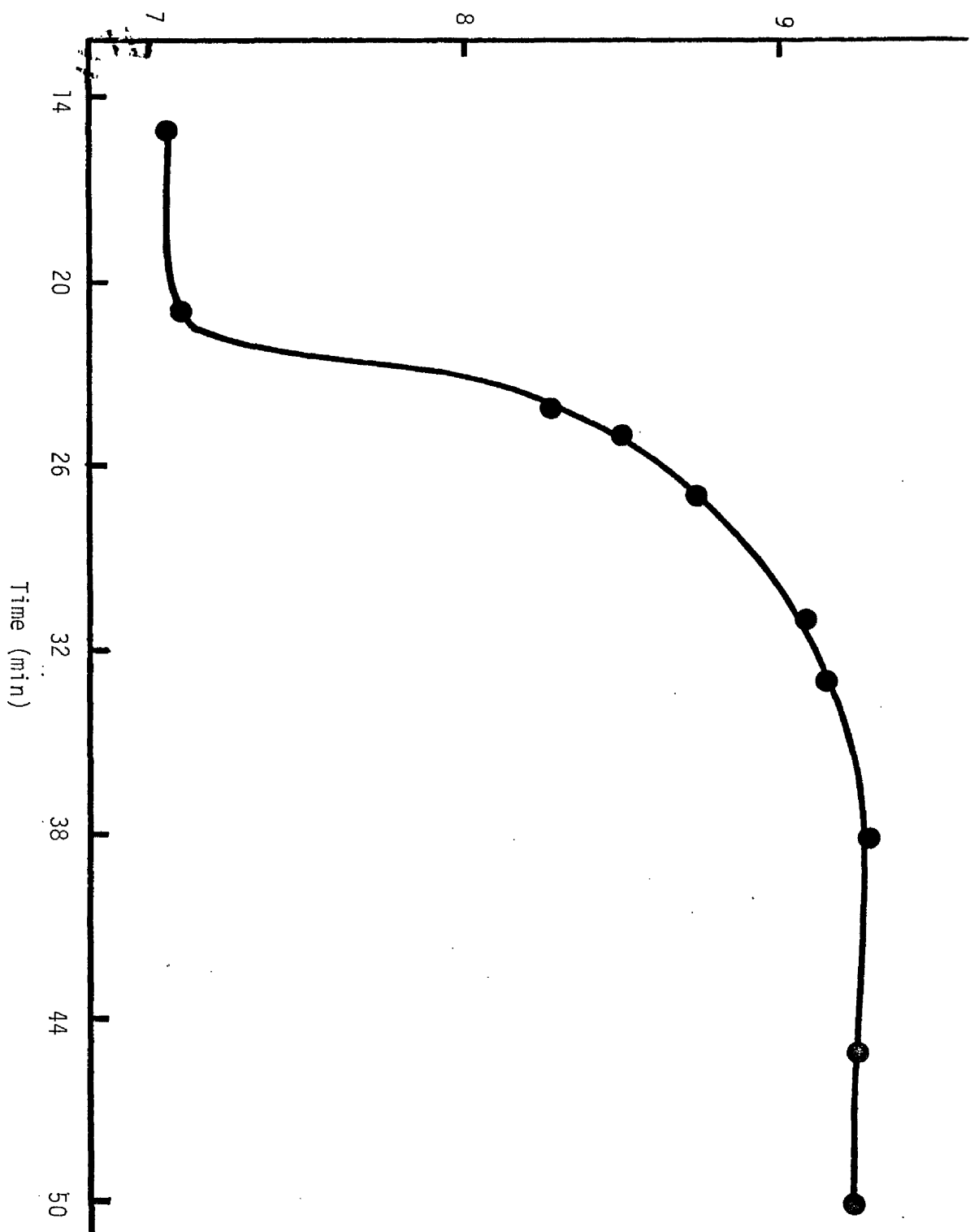


Figure 1.3 One step growth curve for  $\phi C2(W)$  on *Str. lactis* C2 in PLGYG broth at 30°C

the strains studied ~~has~~ been reported as hosts for the temperate phage resident in C2 (McKay and Baldwin, 1973). Because there is no literature available describing common phages for C2 and any of the strains used in the combinations these cultures are probably phage-unrelated. However, the term phage-unrelated is used only to describe the phage relationship existing between the component strains of each culture and  $\phi$ C2(W).

#### Effect of infecting paired phage-unrelated cultures containing

##### *Str. lactis* C2 with $\phi$ C2(W)

The results of a typical experiment are shown in Table 1.3 and reveal that acid production was inhibited for six of the eight paired-strain combinations. Acid production was only slightly affected for two cultures, AM2 + C2 and US3 + C2. That phage infection did not inhibit acid production by the component strains of the cultures shown in Table 1.3 is apparent from the results presented in Table 1.4. The pH attained in the infected AM2 + C2 and US3 + C2 cultures at 6 h is compatible with that attained when a 1% inoculum of US3 or AM2 was used. The pH at 6 h in RSM inoculated with 1% AM2 or US3 was about 5-5.1. It is also apparent from Table 1.3 that extensive replication of  $\phi$ C2(W) occurred in all infected cultures.

The data shown in Table 1.3 were reproducible providing that the initial phage level was carefully controlled and similar inoculum rates were used. If high concentrations ( $>10^6$  pfu/ml) of  $\phi$ C2(W) were used to infect cultures of C2 + C10, significant inhibition of acid production was not obtained.

In some experiments, the total viable count of starter organisms was determined on some of the infected cultures which showed marked inhibition of acid production. The data presented in Table 1.5 reveal

Table 1.3

Effect of infecting paired phage-unrelated single-strain starters with  $\phi$ C2(W)

Culture	pH at 6 h		pfu/ml <sup>(1)</sup> at 6 h ( $\times 10^8$ )
	-phage	+phage	
C2 + C10	4.83	6.16	98
C2 + ML8	4.85	6.22	117
C2 + SC1	5.0	6.15	133
C2 + C6	4.91	6.18	153
C2 + AM2	4.79	4.98	276
C2 + SK11	4.88	5.63	81
C2 + E8	5.13	5.78	79
C2 + US3	4.93	5.25	61

<sup>(1)</sup> Sufficient phage was added to give  $1.3 \times 10^4$  pfu/ml.

Table 1.4

Effect of infecting single-strain cultures of lactic streptococci  
with  $\phi$ C2(W)

Strain	pH at 6 h <sup>(1)</sup>	
	-phage	+phage <sup>(2)</sup>
C2	4.92	6.36
C10	4.83	4.82
ML8	4.88	4.88
E8	5.07	5.10
SC1	5.6	5.6
US3	5.08	5.07
C6	4.95	4.95
SK11	4.96	4.94

(1) Strains were inoculated (2% v/v) into sterile RSM (10% T.S.) and incubated at 30°C.

(2) Sufficient phage was added to give  $1.4 \times 10^4$  pfu/ml.

Table 1.5

Total viable counts in phage-infected paired-strain cultures

Phage-infected culture	pH at 6 h	cfu/ml at 6 h ( $\times 10^6$ )
C2 + C10	6.2	10.0
C2 + C6	6.2	0.57
C2 + ML8	6.16	30



that the cell numbers in infected cultures were low after 6 h incubation. Colony forming units in control cultures were in the region of  $1-3 \times 10^9$  cfu/ml.

The results of the previous experiment bear a marked similarity to the 'nascent phage phenomenon' reported by Anderson and Meanwell (1942), Nichols and Wolfe (1945), Collins (1952a) and Whitehead *et al.* (1952).

Whitehead *et al.* (1953) have suggested that this phenomenon was due to the adsorption of phage to non-homologous strains present in the phage-infected culture. Although these strains did not propagate the phage, the adsorbed phage was thought to disrupt host metabolism, a process presumably leading to cell death. To determine whether adsorption of phage to component strains present in the cultures could be a factor involved in the inhibition observed in the previous experiment the ability of  $\phi C2(W)$  to adsorb to various lactic streptococcal strains was determined (Table 1.6). It is apparent that  $\phi C2(W)$  adsorbed to strains other than those on which it replicated. The phage did not adsorb to SCI and did not adsorb efficiently to E8. Since paired-strain cultures containing SCI and E8 were inhibited by  $\phi C2(W)$  adsorption of  $\phi C2(W)$  did not appear to be a prerequisite for the inhibition of the phage-infected cultures shown in Table 1.3.

Naylor and Czulak (1956) suggested that the production of phage-associated lysin by phage-infected starter cultures was responsible for the nascent phage phenomenon. An attempt was made to detect lytic activity distinct from particulate phage in  $\phi C2(W)$  lysates.

#### BACTERIOLYTIC ACTIVITY OF $\phi C2(W)$ LYSATES

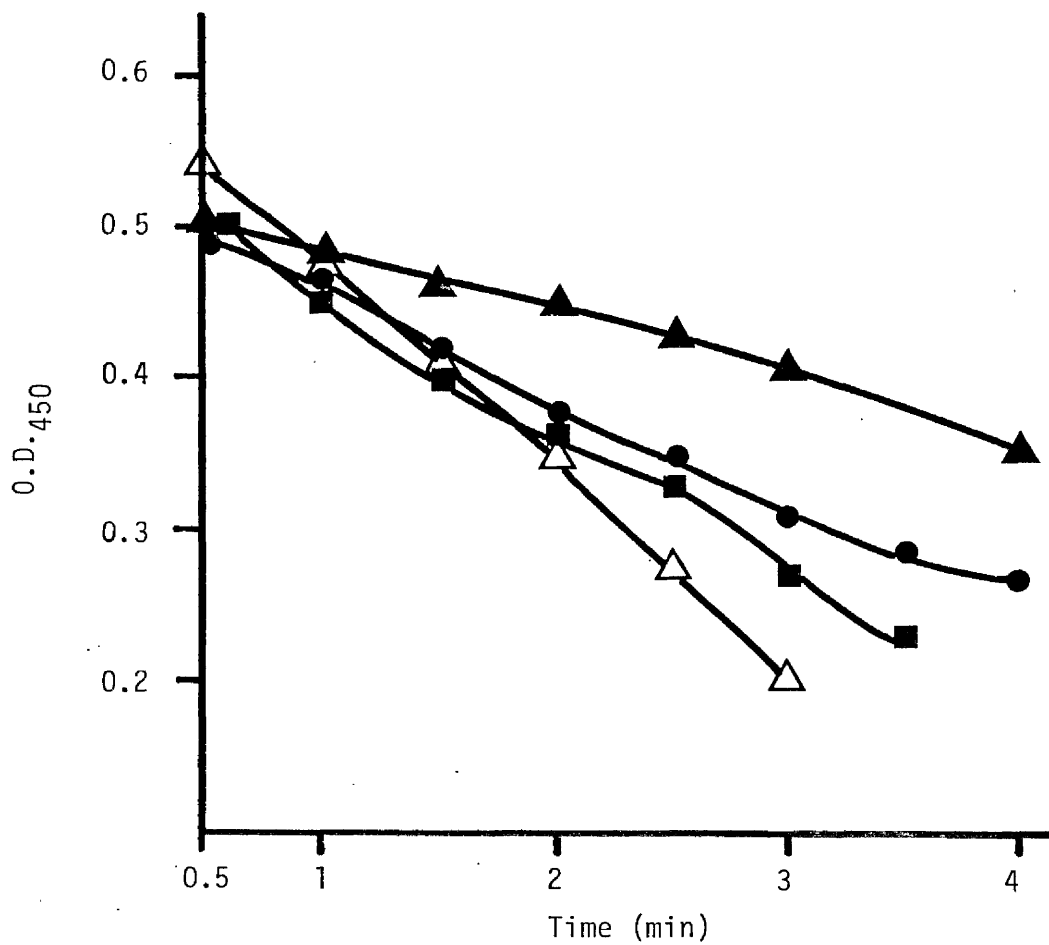
The activity of a  $\phi C2(W)$  lysate containing about  $5 \times 10^{10}$  pfu/ml towards lactic streptococci is shown in Figure 1.4. A marked decrease

Table 1.6

Adsorption of  $\phi$ C2(W) by some lactic streptococci

Strain	% Adsorption
C2	99
E8	14
ML8	27
C10	54
SC1	0

Figure 1.4      Lytic activity of a  $\phi$ C2(W) lysate of  
*Str. lactis* C2 towards lactic streptococci



- ▲-, *Str. lactis* C2
- △-, freeze-dried cells of C2
- , *Str. cremoris* E8
- , *Str. cremoris* 1249

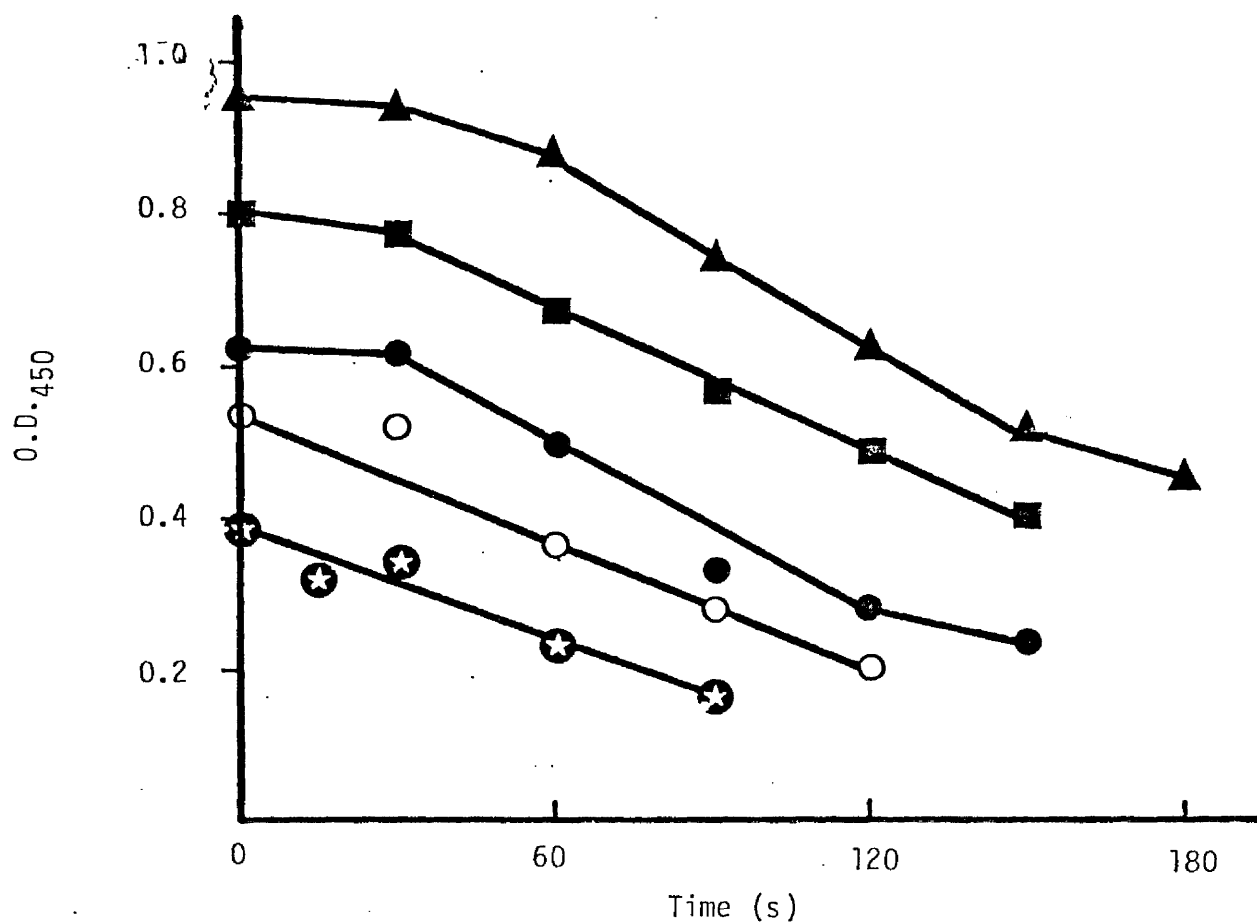
in O.D. with time was observed with all strains. This decrease did not occur in controls in which phage lysate was not added or when heat-treated lysate (90°C for 5 min) was used. Examination of cell suspensions, incubated with lysate for 1 h at 37°C, using phase contrast microscopy revealed extensive cellular lysis confirming that the decrease in O.D. was due to cell lysis. Cells in the process of lysis were also observed. The initially phase bright cells, increased in size, lost refractility and merged almost completely into the background leaving a faint 'ghost' like remnant. Rapid dissolution of chains into collections of single cocci was also a characteristic of the action of  $\phi$ C2(W) lysates on cell suspensions.

The lysate used gave an activity of 150 u/ml when assayed for lytic activity with freeze-dried C2 cells as described on p24. The results show that lytic activity was observed against the producer strain and also against strains which did not propagate  $\phi$ C2(W). That freeze-drying markedly increased the sensitivity of C2 to the lytic agent is also apparent.

#### Assay of lytic activity

The effect of substrate (cell) concentration on the lytic activity of a diluted partially purified preparation of the lytic agent present in  $\phi$ C2(W) lysates is shown in Figure 1.5. Because the slopes of the linear regions of the graphs are almost parallel, the results suggest that the assay of lytic activity is not substrate limited for cell concentrations equivalent to an O.D.<sub>450</sub> of 0.4 or higher. However, more detailed work (Table 1.7) suggested that the assay of lytic activity was substrate limited up to a cell concentration equivalent to an O.D.<sub>450</sub> of about 0.6. Because of this finding all assays were performed using cell suspensions adjusted to give an initial O.D.<sub>450</sub> reading of at least 0.62. That the assay

Figure 1.5 Effect of substrate concentration in the lysis of *Str. lactis* C2 by a  $\phi$ C2(W) lysate



Freeze-dried cells of *Str. lactis* C2 were suspended in 0.1 M K-phosphate buffer, pH 6.7 to give suspensions with the  $O.D._{450}$  values shown. Five mls of cell suspension were mixed with 1 ml of a  $\phi$ C2(W) lysate and the rate of cell lysis at 37°C measured

Table 1.7

Effect of substrate concentration on the  
activity of  $\phi$ C2(W) lysin

Initial O.D. <sub>450</sub>	Enzyme activity ( $\mu$ /ml)
0.39	145
0.54	155
0.62	210
0.8	190
0.95	215

Freeze-dried *Str. lactis* C2 cells were suspended in 0.1 M K-phosphate buffer, pH 6.7 to give the optical densities indicated and were used to titre a partially purified  $\phi$ C2(W) lysin preparation.

gave a linear response between concentration of lytic agent and lytic activity is shown in Figure 1.6.

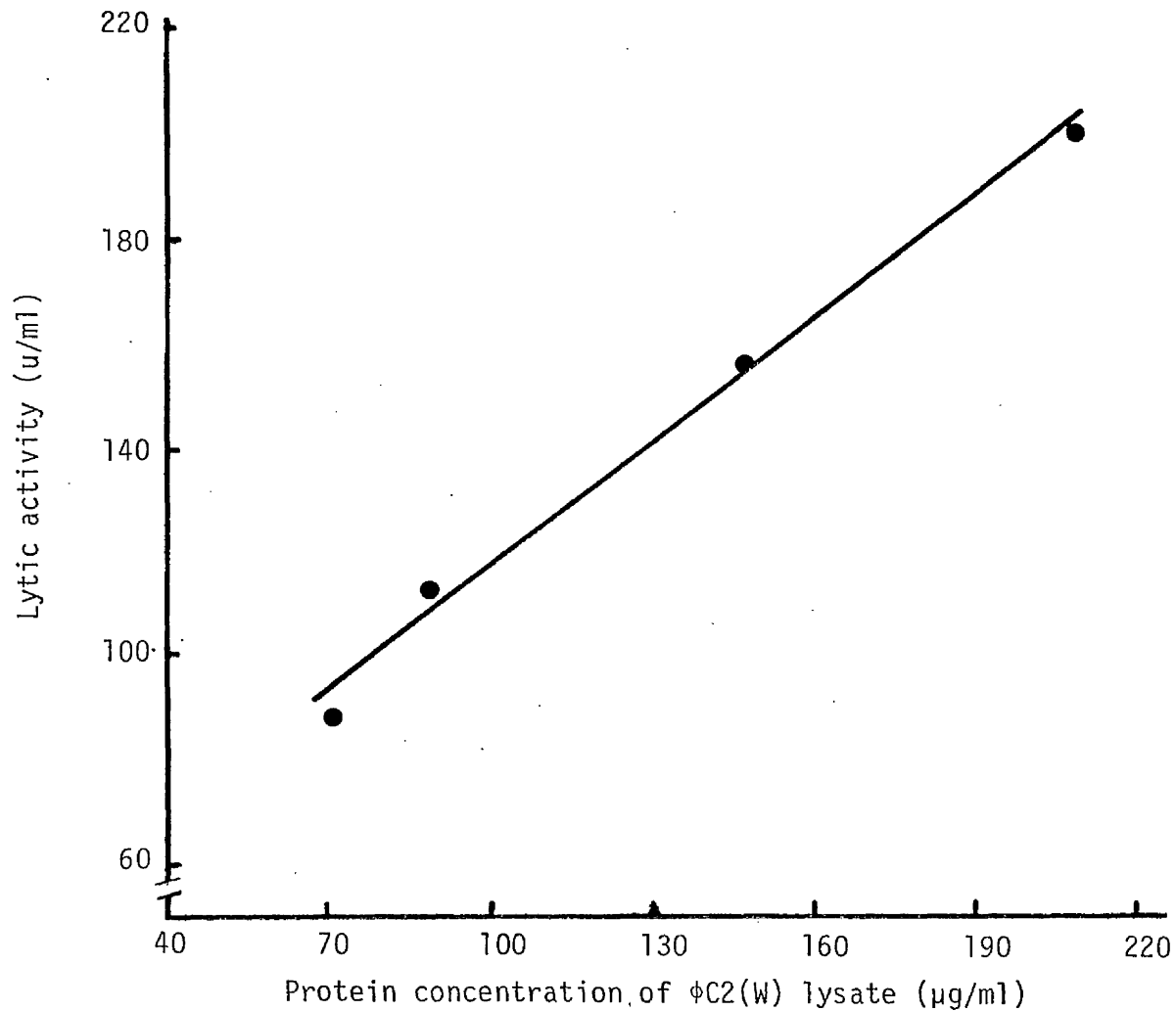
#### Relationship between phage and lytic activity in $\phi$ C2(W) lysates

If  $\phi$ C2(W) is involved directly in the lytic activity of phage lysates towards strains which do not propagate this phage (non-homologous strains) a good correlation should exist between phage and lysin levels in broth lysates. To test this hypothesis lysates were produced containing differing phage concentrations by varying the initial phage to coccus ratio (multiplicity of infection - MOI). A relationship between phage and lysin levels was found (Table 1.8); the higher the phage level, the higher the concentration of lytic activity. However, a proportionate increase in lysin concentration was not found. This finding suggests that the lytic activity of  $\phi$ C2(W) lysates towards non-homologous strains is not directly due to  $\phi$ C2(W).

#### Effect of phage removal on the lytic activity of $\phi$ C2(W) lysates

If the lytic activity of phage lysates is directly associated with the presence of particulate phage, phage removal should reduce lytic activity. Two methods of phage removal were used, namely, acetone precipitation and high speed centrifugation. Although both methods removed more than 95% of the phage present appreciable concentrations of phage remained (Table 1.9). Centrifugation was particularly effective but high centrifugal forces and long centrifugation times were required. Since the lytic activity of  $\phi$ C2(W) lysates was not significantly affected by either method of phage removal, it is apparent that the lytic agent present in lysates is distinct from phage. Furthermore the results indicate that the lytic agent has a considerably lower particle weight.

Figure 1.6 Relationship between lysin concentration and lytic activity



A ϕC2(W) lysate was concentrated and desalted by ultrafiltration using the Amicon YM30 membrane. The concentrate was diluted with 0.05 M K-phosphate buffer to give a series of lysin containing solutions of differing protein content. Protein and lytic activity were determined for each solution.



Table 1.8

Phage and lysin levels in PLGYG broth lysates

Phage (pfu/ml)	Lysin ( $\mu$ /ml)
$3 \times 10^9$	60
$3 \times 10^{10}$	100
$7 \times 10^{10}$	148

A series of  $\phi$ C2(W) - C2 PLGYG broth lysates were produced, in which the initial phage-coccus ratio was varied, to study the relationship between phage and lysin concentration in  $\phi$ C2(W) lysates.

Table 1.9

Effect of acetone precipitation<sup>(1)</sup> and high speed centrifugation<sup>(2)</sup> on phage and lysin levels in  $\phi$ C2(W) lysates

	Volume (ml)	Phage (pfu/ml)	Lytic activity (u/ml)	Total phage (pfu)	Total lytic activity (u)	% phage removed	% activity remaining
<u>Acetone Precipitation</u>							
Lysate	44	$3 \times 10^9$	60	$132 \times 10^9$	2640	-	-
Filtrate	58	$11 \times 10^7$	44	$6.4 \times 10^9$	2552	95	96.7
<u>Centrifugation</u>							
Lysate	50	$7 \times 10^{10}$	140	-	-	-	-
Supernatant	-	$10 \times 10^7$	140	-	-	99.9	-

(1) Phage was precipitated by the addition of 40% (v/v) acetone.

(2) Phage was sedimented at 75,000 x g for 4 h.

### Effect of pH on the lytic activity of $\phi$ C2(W) lysates

Adjustment of lysates (with dilute lactic acid) to pH values of 4.5 or less resulted in appreciable loss of lytic activity. Irreversible loss of activity occurred at pH values of 3.0 or less.

### ATTEMPTS TO INDUCE THE LYSIS-FROM-WITHOUT OF *Str. lactis* C2 WITH HIGH CONCENTRATIONS OF $\phi$ C2(W)

It is generally accepted that lactic streptococcal phages infect host bacteria after first breaking down the cell wall with a lytic enzyme which is associated with the tail of the phage (Reiter, 1973; Law and Sharpe, 1978). In order to obtain further information concerning the nature of the lytic activity present in  $\phi$ C2(W) lysates, attempts were made to determine whether particles of this phage were lytic *per se* i.e. whether  $\phi$ C2(W) possessed a lytic enzyme or not. One method of obtaining this information is to determine whether a phage can induce lysis-from-without (Tsugita, 1971). Although lysis-from-without has been demonstrated frequently with Gram-negative bacteria, comparatively few accounts are available documenting this phenomenon for Gram-positive organisms.

The first report of the lysis-from-without of Group N streptococci by phage was described by Oram and Reiter (1965). These workers working with a lysin-producing phage,  $\phi$ ML3, reported lysis-from-without of several lactic streptococci in 0.1 M potassium phosphate buffer, pH 6.7 with a MOI of 13.5.

Before these experiments were undertaken it was necessary to have high titre phage preparations. Because earlier experiments suggested that the lytic activity present in  $\phi$ C2(W) lysates may be due to an agent other than phage, partially purified preparations, essentially free of

medium constituents, were required. Alternatively, a method which selectively removes lytic agents other than phage could be used.

One method of phage purification involves high speed centrifugation. Unfortunately a high speed centrifuge was not available at this time and alternative methods for producing high titre phage preparations free of possible contaminating lytic activity from substances present in broth lysates were investigated.

Preliminary experiments established that high titre preparations of  $\phi$ C2(W) could be obtained which were apparently free of lytic activity.

Preparations free of lytic activity were produced using three methods:

- (i) Ultrafiltration
- (ii) Negative chromatography with a dye-ligand
- (iii) Heat treatment.

The  $\phi$ C2(W) preparation produced by each method was assayed for lytic activity using freeze-dried C2 cells suspended in 0.1 M K-phosphate buffer, as described on p24.

Details of the production methods and results are shown in Tables 1.10, 1.11 and 1.12. Both crude and partially purified broth lysates were used in these experiments. The material used to obtain the data shown in Tables 1.10 and 1.11 was a partially purified broth lysate which contained both high levels of phage and lytic activity.

The results clearly show that high titre phage preparations did not exhibit lytic activity against C2 cells suspended in buffer although high multiplicities of infection were used.

Table 1.10

Removal of  $\phi$ C2(W) lysin and partial purification of  $\phi$ C2(W) by ultrafiltration<sup>(5)</sup>

Material	Protein (mg/ml)	Volume (ml)	Phage (pfu/ml)	Lysin activity <sup>(2)</sup> (u/ml)	Specific activity		Purification	Phage yield
					Phage (pfu/ml)	Protein (mg/ml)		
Partially purified (1) $\phi$ C2(W) lysin	0.2	200	$5.3 \times 10^{10}$	800	$26.5 \times 10^9$	-	-	-
Washed concentrate <sup>(3)</sup>	2.0	4	$7.3 \times 10^{13}$	<1 <sup>(4)</sup>	$3.65 \times 10^{13}$	1,377	275	

- (1) Partially purified lysin preparation obtained by ion exchange chromatography on Amberlite CG50 (pI25).
- (2) Assayed using freeze-dried C2 cells in 0.1 M K-phosphate buffer pH 6.7 at 37°C.
- (3) Washed with quarter strength Ringer solution until all traces of lytic activity were removed (x 6 washes).
- (4) C2 cells were infected with a MOI of 3,968.
- (5) Ultrafiltration was undertaken using an Amicon Model 202 stirred cell fitted with a XM300 membrane.

Table 1.11

Removal of  $\phi$ C2(W) lysin and partial purification of  $\phi$ C2(W) using the dye ligand Green A

Material	Protein (mg/ml)	Volume (ml)	Phage (pfu/ml)	Lysin activity (u/ml)	Specific activity $\frac{\text{Phage (pfu/ml)}}{\text{Protein (mg/ml)}}$	Purification	Phage yield
Partially purified $\phi$ C2(W) lysin <sup>(1)</sup>	0.2	2	$5.3 \times 10^{10}$	800	$26.5 \times 10^{10}$	-	-
Column effluent	0.1	2	$4.1 \times 10^{10}$	$< 1^{(3)}$	$41 \times 10^{10}$	1.6	77.4

<sup>(1)</sup>Partially purified lysin preparation obtained by ion exchange chromatography on Amberlite CG50 (p125).<sup>(2)</sup>Assayed using freeze-dried C2 cells in 0.1 M K-phosphate buffer pH 6.7 at 37°C.<sup>(3)</sup>C2 cells infected with a MOI of about 100.

Table 1.12

Inactivation of  $\phi$ C2(W) lysin by heat treatment

Material	Phage (pfu/ml)	Lysin activity (u/ml)	Phage yield (%)
Broth lysate	$4.1 \times 10^{10}$	120 <sup>(1)</sup>	-
Lysate treated for 1 min at 57°C	$2.3 \times 10^{10}$	<1	-
Lysate treated for 4 min at 57°C	$4.0 \times 10^{10}$	<1 <sup>(2)</sup>	98

(1) Assayed using freeze-dried C2 cells in 0.1 M K-phosphate buffer, pH 6.7 at 37°C.

(2) C2 cells infected with a MOI of 66.7.

In addition it is also apparent that:

(1)  $\phi$ C2(W) is markedly larger than the agent responsible for lytic activity; lytic activity can be 'washed' out of phage preparations during ultrafiltration (Table 1.10).

(2) the chromatographic properties of the phage and the agent responsible for lytic activity in phage lysates on the dye ligand Green A were different; the lytic agent was bound but the phage was not bound (Table 1.11).

(3) the phage was considerably more heat stable than the lytic agent (Table 1.12).

These results provide further evidence that the lytic agent present in  $\phi$ C2(W) lysates is distinct from phage. It is of interest to note that excellent purification of  $\phi$ C2(W) was obtained by ultrafiltration. Significant purification was also obtained by dye ligand chromatography. The purification data presented in Table 1.11 are slightly misleading since the 2 ml sample applied to the Dyematrix column (gel volume only 2 ml) was almost at the limit of the adsorption capacity of the column. When smaller sample volumes (0.5 ml) were applied, considerably higher purification figures (approaching 100-fold purification) were obtained.

Although Reiter and Møller-Madsen (1963) clearly stated in their review that calcium ions were not essential for the adsorption of phage to Group N streptococci and that irreversible adsorption occurred efficiently in the presence of monovalent cations, several authors of review articles (Babel, 1962; Law and Sharpe, 1978) have reported that calcium was required for adsorption. Since one explanation for the failure to demonstrate lysis-from-without with  $\phi$ C2(W) could have been the inability of this phage to adsorb to *Str. lactis* C2 in phosphate buffer, the effect of suspension



medium on phage adsorption (Table 1.13) was studied. It was found that adsorption occurred efficiently in either 0.1 M phosphate buffer or in PLGYG supplemented with  $\text{Ca}^{++}$ . These results clearly disprove the hypothesis that the failure of  $\phi\text{C2(W)}$  to lyse *Str. lactis* C2 was due to the inability of  $\phi\text{C2(W)}$  to adsorb to its host in phosphate buffer. In a further attempt to obtain evidence concerning the lytic activity of lysin free preparations of  $\phi\text{C2(W)}$  the effect of infecting viable and heat-killed C2 cells suspended in calcium-supplemented PLGYG with a phage:coccus ratio of 10,000:1 was studied and is shown in Figure 1.7. Prior to phage infection the untreated C2 cells were growing normally. Growth, however, ceased following infection. The drop in optical density which occurred was due to the dilution of the 1.5 ml of cells in the cuvette with 0.45 ml of the partially purified phage preparation. The preparation was obtained using the procedure shown in Table 1.10 and exhibited little adsorption at 450 nm. A similar drop in O.D. was obtained when distilled water was used. Lysis of the infected, un-heated, cells did not occur until about 15 min and was complete at 36.5 min. These results approximately parallel the data shown in the one-step-growth experiment (Figure 1.3), and show that the infection of *Str. lactis* C2 with high titre preparations of  $\phi\text{C2(W)}$  does not result in lysis from without. This statement is supported by the failure of high concentrations of  $\phi\text{C2(W)}$  to demonstrate lytic activity against heat-killed cells of *Str. lactis* C2.

#### LYTIC ACTIVITY IN FILTRATES OF BROKEN AND UNBROKEN CELLS OF *Str. lactis* C2 INFECTED WITH $\phi\text{C2(W)}$

Attempts were made to determine whether the lytic activity in  $\phi\text{C2(W)}$  lysates was phage induced. The lytic activity in C2 cells infected with  $\phi\text{C2(W)}$  at 30°C and broken by sonication at 5°C is shown in Figure 1.8. Lytic activity was detected in broken infected cells after 10 min, this

Table 1.13

Effect of medium on the adsorption of  $\phi$ C2(W) by *Str. lactis* C2

Medium	% Adsorption
PLGYG	99.0
0.1 M potassium phosphate	98.9

Figure 1.7      Effect of infecting viable and heat-killed cells of *Str. lactis* C2 with  $\phi$ C2(W) using a phage:coccus ratio of 10,000

PLGYG broth was inoculated with log phase cells of *Str. lactis* C2 to give the O.D. shown and was supplemented with 10 mM  $\text{Ca}^{++}$ . After 10 min incubation at 30°C, a partially purified  $\phi$ C2(W) preparation was added to give a phage to coccus ratio of 10,000:1. Cultures were incubated at 30°C.

—●— , viable cells

—○— , cells of C2 which had been killed by heating to 70°C for 20 min

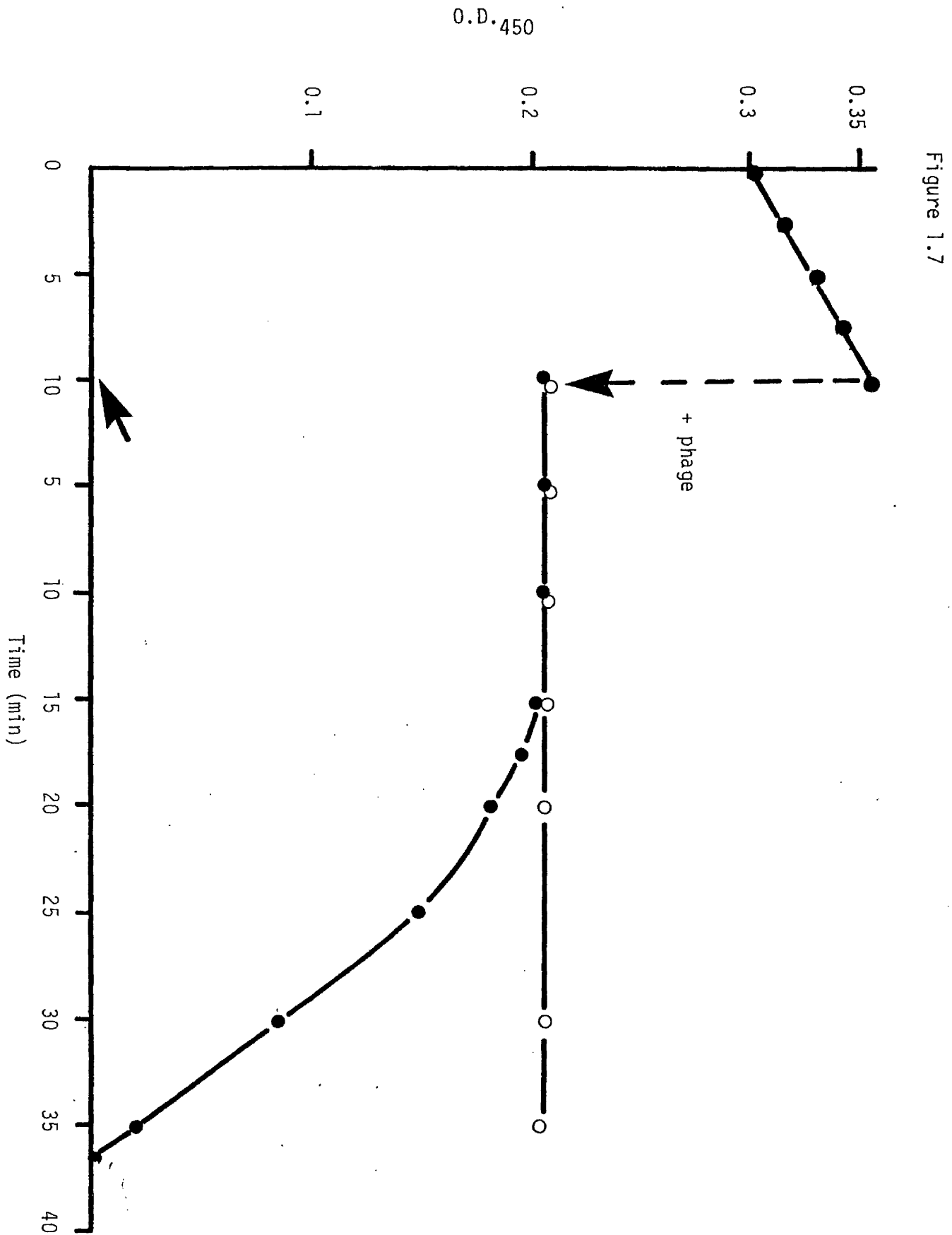
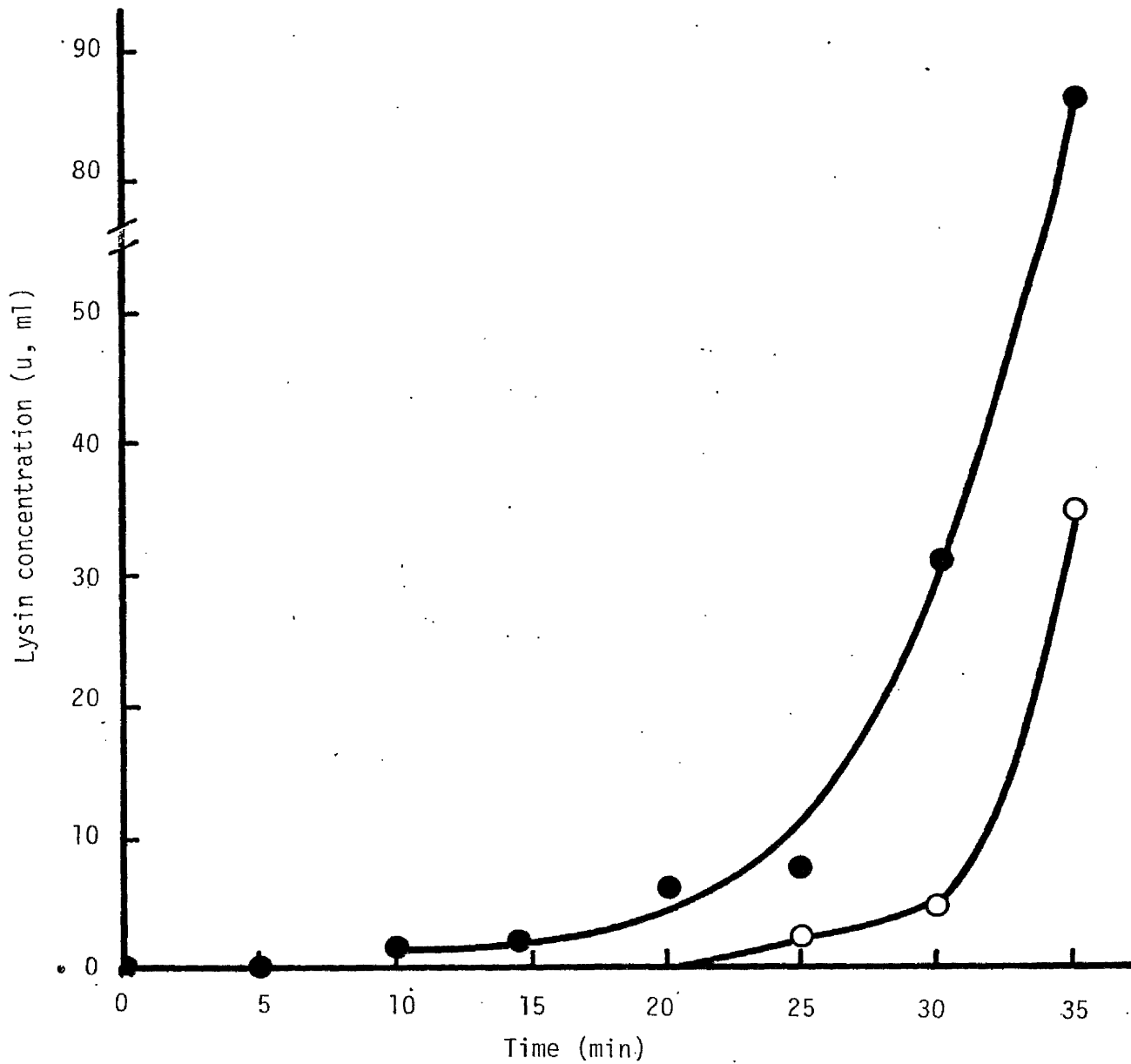


Figure 1.8 Lysin production by cells of *Str. lactis* C2 infected with  $\phi$ C2(W)



- , lytic activity in the cell-free culture broth.  
Cells broken by sonic disruption
- , lytic activity in the cell-free culture broth.  
Cells not broken by sonic disruption

is about halfway through the latent period and increased exponentially thereafter reaching a maximum concentration, in this experiment, of about 90 u/ml at 35 min. In repeat experiments in which multiplicities of infection of 1, 10 and 40 were used no significant differences in the general pattern of lysin production depicted in Figure 1.8 were found. The concentration of lysin found in each experiment varied, however. Lysin activity was not found in the supernatant of unbroken infected cells until the end of the latent period. The concentration increased thereafter reaching a high concentration at the rise period. Lytic activity was found only in C2 cells infected with  $\phi$ C2(W). These results show clearly that the lytic activity present in lysates of C2 is induced by this phage. Because of the correlation between the time required to detect lytic activity in unbroken phage-infected cells and the latent period the data indicate that lytic activity is released at the time of cellular lysis. This statement is supported by the demonstration of lytic activity in broken  $\phi$ C2(W)-infected C2 cells prior to the latent period.

*Str. lactis* C2 is also host to  $\phi$ 712. When  $\phi$ 712 lysates of C2 were tested for lytic activity against washed log phase cells of C2 and C10 no activity was detected (Table 1.14). The activity of  $\phi$ 712 lysates against freeze-dried cells of C2, SC1, U.G. 1249 and E8 was also determined. No lytic activity was found against C2 or SC1 but very slight activity was found when 1249 and E8 were used. This observation provides further evidence that the lytic activity present in  $\phi$ C2(W) lysates of *Str. lactis* C2 is induced by  $\phi$ C2(W).

It has been suggested (Reiter, 1973) that phages for the lactic streptococci infect host bacteria after first breaking down the cell wall with phage tail lysin to permit DNA introduction. Since the previous results clearly suggested that  $\phi$ C2(W) particles did not possess lytic

Table 1.14

Bacteriolytic activity of  $\phi$ 712 lysate of *Str. lactis* C2  
against various Group N streptococci

Strain	Lysin activity (u/ml) <sup>(1)</sup>
(1) F.D. C2	<1
F.D. E8	2.6
F.D. 1249	4
F.D. SC1	<1
C2	<1
C10	<1

F.D. Freeze-dried log phase cells.

(1) Cells were suspended in 0.1 M K-phosphate buffer, pH 6.7 at 37°C as described above in Methods.

(2) Lysate contained  $6.5 \times 10^9$  pfu/ml of  $\phi$ 712 when assayed using *Str. lactis* C2.

activity attempts were made to liberate lytic activity from high titre lysin-free preparations of  $\phi$ C2(W).

Oram and Reiter (1965) found sonication to be particularly effective for liberating lytic activity from high titre preparations of  $\phi$ ML3. These workers claimed to demonstrate the release of lytic activity by sonication from preparations of  $\phi$ ML3 containing  $3.6 \times 10^{11}$  pfu/ml. Using sonication conditions similar to those described by the previous workers and  $\phi$ C2(W) preparations ranging in titre from  $5 \times 10^{10}$  -  $1 \times 10^{13}$  pfu/ml the author could not demonstrate release of lytic activity. Sonication, however, markedly increased the viscosity of the phage preparation, presumably due to the release of phage DNA. Since phage lysins are highly basic proteins and DNA is highly acidic it is not surprising that Tsugita (1971) reported that some phage lysins may be inactivated by interaction with DNA. It was considered unlikely that a lysin-phage DNA interaction was responsible for the failure to demonstrate lysin release from  $\phi$ C2(W) particles since preparations of purified and unpurified lysin could be sonicated without significantly affecting lysin activity. Nevertheless further experiments were undertaken in which sonication was performed in media containing added DAE cellulose, an agent which binds DNA and other negatively charged species.

Preliminary experiments established that neither phage nor lysin bound to DAE cellulose adjusted to pH values in the range 6.7-8.0 and equilibrated with 0.01 M K-phosphate buffer. Sonication of high titre phage preparations in the presence of DAE cellulose adjusted to pH 6.7 also failed to demonstrate the release of lytic activity from  $\phi$ C2(W) particles.



## INHIBITORY MECHANISM INVOLVED IN THE INHIBITION OF PAIRED-STRAIN CULTURES BY $\phi C2(W)$

In this section an attempt is made to elucidate the mechanism involved in the inhibition of acid production previously reported (p41) when paired-strain cultures containing C2 were infected with  $\phi C2(W)$ .

The previous experiments have established that cells of C2 infected with  $\phi C2(W)$  produce a lytic agent, distinct from phage, which lyses strains of lactic streptococci regardless of whether they propagate  $\phi C2(W)$ . Based on these findings the following hypothesis is offered as an explanation for the nascent effects experienced with  $\phi C2(W)$ . When a culture containing C2 and a strain which does not propagate  $\phi C2(W)$  is infected with  $\phi C2(W)$ , the infected cells of C2 lyse, and release a phage-induced lytic enzyme, hereafter referred to as phage lysin or lysin. The lysin lyses the phage-resistant strain and it is the lysis of this strain which is responsible for the inhibition of acid production experienced when paired-strain cultures containing C2 are infected with  $\phi C2(W)$ .

One method of testing this hypothesis would be to isolate a mutant of  $\phi C2(W)$  which does not induce C2 to produce lysin. If paired-strain cultures containing C2 were infected with this mutant phage and inhibition of acid production was not found, this would provide evidence for the hypothesis.

Since phages which induce host-strains to produce phage lysin can be identified by their ability to produce haloes around plaques (Tsugita, 1971) attempts were made to isolate a phage mutant which did not exhibit haloes around plaques. Over a period of three years a large number of phage plates were routinely examined for evidence of a 'haloless' mutant but with no success. Since a mutant of  $\phi C2(W)$  was not available,

experiments were undertaken using  $\phi 712$ . This phage does not induce host strains to produce significant levels of phage lysin.

Some characteristics of  $\phi 712$  are listed in Table 1.15. Unlike  $\phi C2(W)$ ,  $\phi 712$  gives minute plaques on C2. The plaques are not surrounded by a halo. Both phages appear to have a similar host range. Unlike  $\phi C2(W)$ ,  $\phi 712$  has an isometric head and a long tail which terminates in a base plate (Plates 1.3a, 1.3b).

The effect of infecting the paired-strain cultures studied previously (p42) with  $\phi 712$  is shown in Table 1.16. Examination of the results reveals that infection with  $\phi 712$  did not markedly inhibit acid production by the paired-strain cultures. The higher pH found in the infected cultures is consistent with the destruction of the C2 component of each combination and the resultant acid being <sup>produced</sup> by a 1% inoculum of the remaining strain.

The results of the previous experiment can be interpreted as providing evidence for the hypothesis.

A second series of experiments ~~was~~ designed to test the hypothesis. In these experiments single-strain cultures of C2 and paired-strain cultures of C2 + C10 were grown in broth media and infected with  $\phi C2(W)$  or  $\phi 712$ . It was envisaged that if a lytic agent was involved in the nascent reaction, overt lysis of paired-strain cultures should be apparent in broth media.

The effect of infecting monocultures of C2 and cultures of C2 + C10 on growth, lysin and phage production is shown in Figure 1.9. Mass lysis of the phage-infected C2 culture had occurred by 2 h incubation. High levels of lysin and phage were found at this time. However, maximum lysin levels were found after mass lysis had occurred. Mass lysis also

Table 1.15

Some properties of  $\phi 712$ 

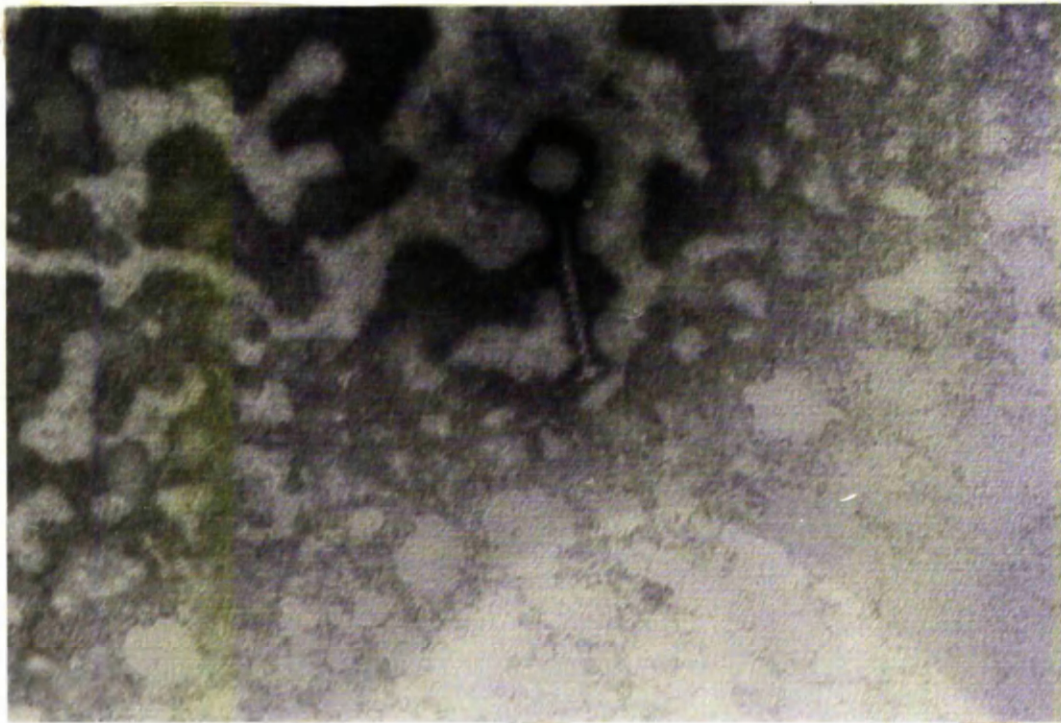
Host range	<i>Str. lactis</i> strains C2, C10 and ML3 <sup>(1)</sup>
Plaque size	Minute
Halo production	-
Head type	Isometric
Head length (nm)	40 x 40 <sup>(2)</sup> ; 45 x 45 <sup>(3)</sup>
Tail length (nm)	140 <sup>(2)</sup> ; 150 <sup>(3)</sup>
Base plate length (nm)	21 <sup>(2)</sup>

(1) Strains ML8, C10, SC1, E8, C6, SK11, AM2, US3, P2 and DRC2 did not propagate  $\phi 712$ .

(2) Determined by the author.  $\phi 712$  propagated on C2. Results are means of five determinations. Because the quality of electron micrographs was very poor, it was difficult to estimate dimensions precisely.

(3) Determined by Davies at NIRD.  $\phi 712$  propagated on 712.

Plate 1.3 Particle morphology of  $\phi 712$



(a)  $\phi 712$  propagated on *Str. lactis* C2 and stained negatively with phosphotungstic acid. Unpurified lysate. x 175,000



(b)  $\phi 712$  propagated on *Str. lactis* 712 and stained negatively with uranyl acetate. Purified using cesium chloride. x 300,000

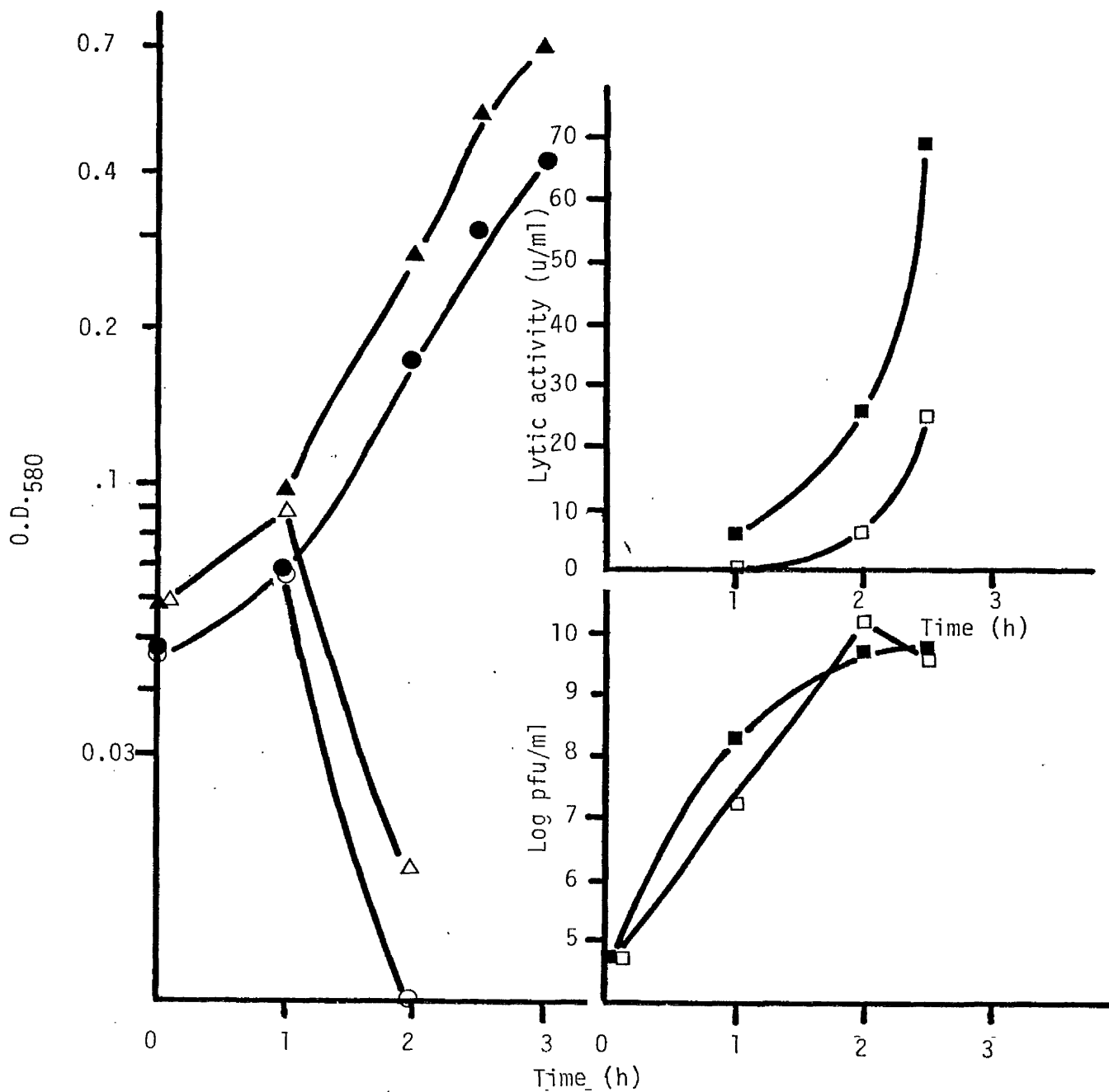
Table 1.16

Effect of infecting paired phage-unrelated single-strain  
starters with  $\phi 712$

Culture	pH after 6 h		pfu/ml at 6 h ( $\times 10^8$ )
	-phage	+phage	
C2 + C10	4.7	4.8	100
C2 + SK11	4.8	5.25	64
C2 + E8	4.7	5.2	113
C2 + US3	4.8	5.1	80
C2 + C6	4.91	5.24	200
C2 + AM2	4.7	5.08	108
C2 + ML8	4.79	5.02	75
C2 + SC1	4.84	5.63	115
C2	4.82	6.21	105

Paired phage-unrelated cultures each containing similar cell numbers were inoculated into RMS (10% T.S.) at the 2% (v/v) level and infected with  $1.4 \times 10^4$  pfu/ml of  $\phi C2(W)$ .

Figure 1.9 Effect of infecting single-strain cultures of *Str. lactis* C2 and paired-strain cultures containing C2 and C10 with  $\phi$ C2(W) on growth, lysin and phage production



Culture growth

$\blacktriangle$  —, C2 + C10;  $\triangle$  —, C2 + C10 +  $\phi$ C2(W);  $\bullet$  —, C2;  
 $\circ$  —, C2 +  $\phi$ C2(W)

Lysin and phage production

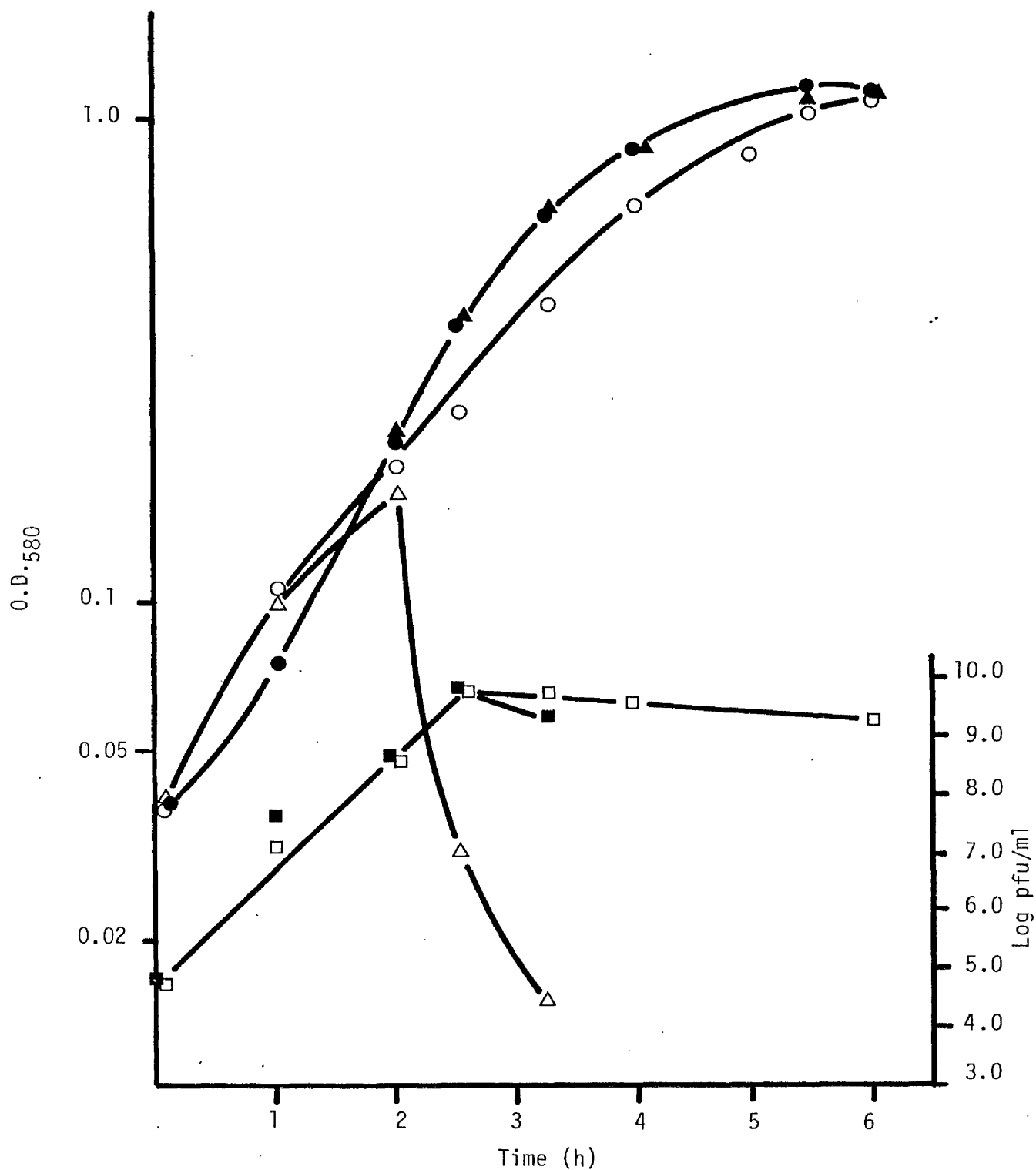
$\blacksquare$  —, C2 +  $\phi$ C2(W);  $\square$  —, C2 + C10 +  $\phi$ C2(W) respectively

occurred in the phage-infected paired-strain culture. Although low levels of lysin (2 u/ml) were detected in this culture prior to mass lysis (1 h), it was generally difficult to detect lysin in  $\phi$ C2(W)-infected cultures of C2 + C10 prior to mass lysis. Lysin levels in the paired-strain cultures were markedly lower than those in the infected monoculture presumably due to the adsorption of lysin to C10.

The effect of infecting monocultures of C2 and cultures of C2 + C10 with  $\phi$ 712 is shown in Figure 1.10. Mass lysis of C2 was apparent after 2.5 h incubation and coincided with the maximum titre of  $\phi$ 712. In contrast with the previous results, the  $\phi$ 712-infected C2 + C10 culture did not show mass lysis. The kinetics of  $\phi$ 712 replication in this culture were similar with those in the monoculture and suggested that the C2 component of the paired-strain culture had been destroyed by phage after 2-2.5 h. This suggestion is supported by the significantly lower optical density values found in the infected paired-strain culture compared with the control. These lower values were apparent after 2 h incubation. The data presented in Figures 1.9 and 1.10 suggest that the inhibition of acid production experienced when cultures of C2 + C10 were infected with  $\phi$ C2(W) was due to the lysis of the component strains. These results also provide evidence for the hypothesis.

Further experiments designed to test the hypothesis that a phage-induced lysin was involved in the inhibition of acid production found in the infected paired-strain cultures were undertaken using lysin-resistant mutants of C2. Lysin-resistant mutants were isolated by plating high numbers of C2 cells onto GMA agar (Limsowtin and Terzaghi, 1976) containing partially purified  $\phi$ C2(W) lysin. The properties of these and other lysin-resistant Group N streptococci are discussed in Chapter 4. The mutants used were resistant to lysin and did not propagate  $\phi$ C2(W) but retained

Figure 1.10 Effect of infecting single-strain cultures of *Str. lactis* C2 and paired-strain cultures containing C2 + C10 with  $\phi 712$  on growth and phage production



- ▲-, turbidity of C2 in PLGYG broth at 30°C
- △-, turbidity of C2 +  $\phi 712$  in PLGYG at 30°C
- , turbidity of C2 + C10 in PLGYG at 30°C
- , turbidity of C2 + C10 +  $\phi 712$  in PLGYG at 30°C
- ,  $\phi 712$  replication in the infected C2 + C10 culture
- ,  $\phi 712$  replication in the infected C2 culture



sensitivity to  $\phi 712$  and had similar rates of acid production in RSM at 30°C to the parent strain. The effect of infecting paired-strain cultures containing C2 and the lysin and phage-resistant mutants is shown in Table 1.17. It is apparent that neither culture was markedly inhibited. The phage titres at 6 h in the infected cultures were lower than those in the experiment shown in Table 1.3. The lower titres were found in all experiments and are probably due to the adsorption of  $\phi C2(W)$  by the mutant strains. Both mutants retained the capacity to bind  $\phi C2(W)$ .

The results of the experiments presented in this section combined with the demonstration of lytic activity present in  $\phi C2(W)$  lysates strongly suggest that it is the bacteriolytic agent produced by  $\phi C2(W)$ -infected C2 cells which is responsible for the inhibition of acid production in paired-strain cultures infected with  $\phi C2(W)$ .

#### EFFECT OF INFECTING MULTI-STRAIN CULTURES WITH $\phi C2(W)$

Multiple-strain cultures are used as starters in the manufacture of cheese and other fermented dairy products. The rationale for their use has been described (p11).

The effect of infecting a multi-strain culture containing C2, C10 and ML8 with two different concentrations of  $\phi C2(W)$  is shown in Figure 1.11. Acid production was inhibited in both infected cultures and shows that  $\phi C2(W)$  can inhibit acid production in multi-strain cultures. Inhibition of acid production was observed after about 3 h incubation; at which time maximum phage levels had been reached. However, acid production continued at a reduced rate and coagulation was evident in both infected cultures when they were examined next morning (further 16 h incubation).

Table 1.17

Effect of infecting paired-strain cultures containing *Str. lactis* C2  
and a lysin and phage resistant variant of C2 with  $\phi$ C2(W)

Culture	pH at 6 h		pfu/ml <sup>(1)</sup> at 6 h ( $\times 10^8$ )
	-phage	+phage	
C2	4.92	6.28	ND
C2 + C2 (LRM1)	4.83	5.09	1.2
C2 + C2 (LRM2)	4.88	5.26	10

(1) Initial phage level was  $1.3 \times 10^4$  pfu/ml.

ND Not determined.

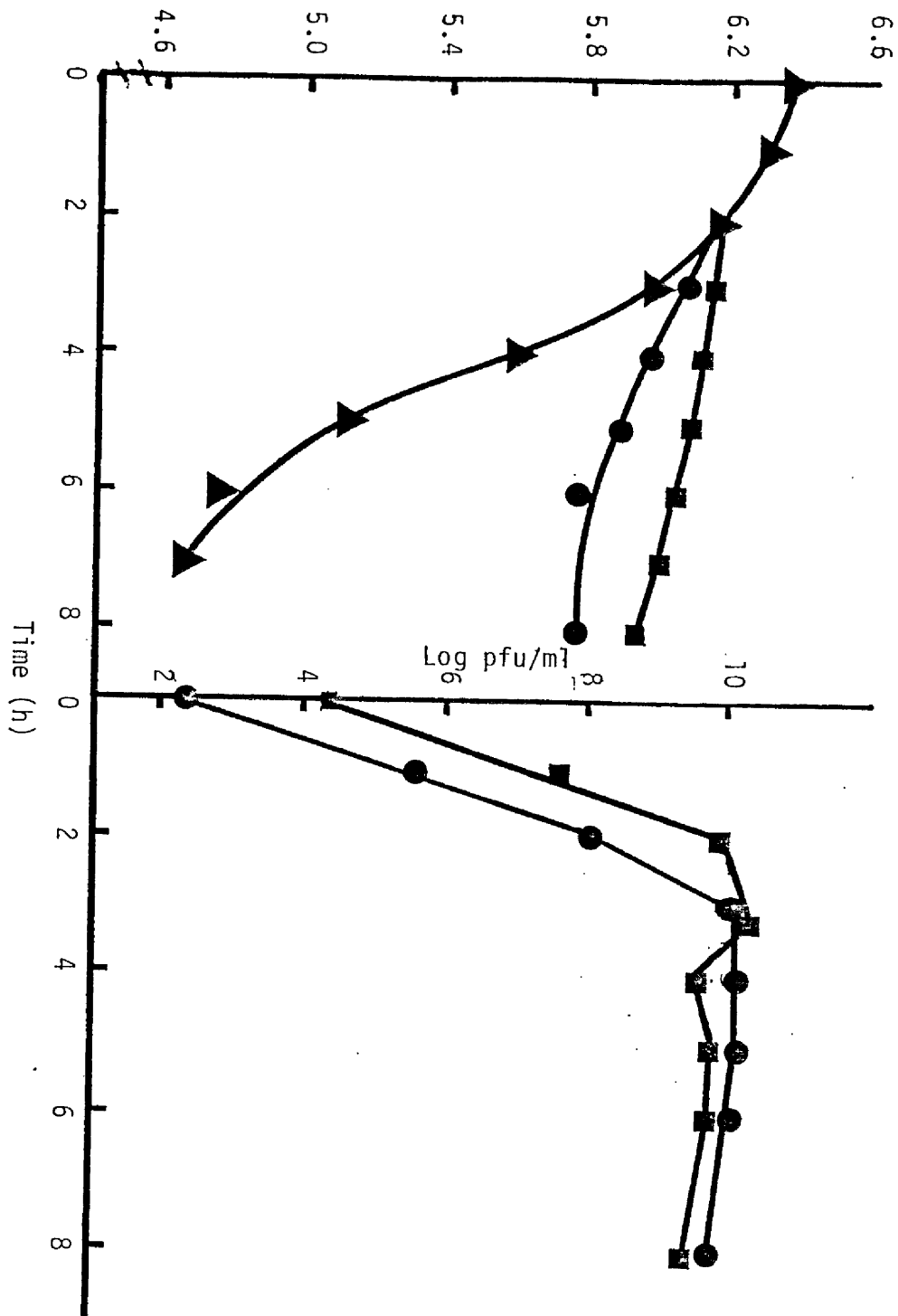
Figure 1.11      Effect of infecting a multi-strain culture containing *Str. lactis* strains C10, ML8 and C2 with  $\phi$ C2(W)

RSM was inoculated with a multi-strain culture containing similar cell numbers of ML8, C2 and C10 and incubated at 30°C.

- ,    acid and phage production respectively in cultures infected with  $2 \times 10^2$  pfu/ml of  $\phi$ C2(W)
- ,    acid and phage production respectively in cultures infected with  $2 \times 10^4$  pfu/ml of  $\phi$ C2(W)
- ▲— ,    acid production in cultures not infected with  $\phi$ C2(W)

pH

Figure 1.11



MINIMUM LEVEL OF *Str. lactis* C2 REQUIRED IN A MULTI-STRAIN  
CULTURE FOR DEMONSTRATION OF NASCENT EFFECTS WITH  $\phi$ C2(W)

The results of a typical experiment (Table 1.18) indicate that inhibition of the multi-strain culture occurred when the initial cell content of C2 was 5% or greater of the total cell population. It is also apparent that the level of inhibition obtained appeared to be related to the initial concentration of C2 cells present. In repeat experiments it was established that the minimum concentration of C2 found necessary for inhibition of acid production above was reproducible. However, significant variation in the level of inhibition obtained was found.

MINIMUM LEVEL OF *Str. lactis* C2 REQUIRED IN A MULTIPLE-STRAIN  
CULTURE TO ENABLE ENUMERATION OF  $\phi$ C2(W) BY THE PLAQUE ASSAY

The previous experiment demonstrated that the infection of a multi-strain culture, containing only low levels of C2, with  $\phi$ C2(W) can result in marked inhibition of acid production. Cheese factories frequently perform phage detection tests on whey from slow vats using as host a sample of phage-free starter. It was considered desirable to obtain information on the level of C2 required in such a culture to demonstrate the presence of phage using the plaque assay method. A slight modification of the method described on p23 was used in these experiments to increase the sensitivity of the method; phage adsorption was allowed to occur at room temperature and not in the agar at 46°C. The value of this modification has been described (Mullan, 1977; Mullan, 1979).

The effect of titrating a  $\phi$ C2(W) preparation against a multi-strain culture (C2 + C10 + ML8) containing different levels of C2 is shown in Table 1.19. Plaque formation was apparent with cultures containing 20% (v/v) C2 although plating efficiency declined with decreasing concentration

Table 1.18

Relationship between the C2 cell content of a multi-strain culture and acid production as revealed by the pH attained after 5 h at 30°C in cultures affected and not infected with  $\phi$ C2(W)

	-Phage				
% C2 in Culture:	1	5	10	20	50
pH at 5 h:	5.61	5.64	5.62	5.35	5.60
	+Phage				
% C2 in Culture:	1	5	10	20	50
pH at 5 h:	5.60	5.86	5.94	5.97	6.11

RSM was inoculated with multi-strain cultures containing *Str. lactis* strains C2, ML8 and C10 which contained different levels of C2. The initial phage level in infected cultures was  $1.4 \times 10^4$  pfu/ml. Initial pH of inoculated milk was 6.5.

Table 1.19

Effect of varying the *Str. lactis* C2 cell content of a multiple-strain starter<sup>(1)</sup> on the titre of  $\phi$ C2(W)

% C2 in Mixture	pfu/ml
5	$<1 \times 10^8$ <sup>(2)</sup>
10	$<1 \times 10^8$ <sup>(2)</sup>
20	$41 \times 10^8$
33	$44 \times 10^8$
100	$58 \times 10^8$

<sup>(1)</sup> Multiple-strain starter contained C10 and M18 in addition to C2.

<sup>(2)</sup> Reduced growth evident; see text for details.

of host cells. No evidence of plaque formation was found in assay media inoculated with cultures containing 5 or 10% (v/v) of C2. However, reduced growth was present in the overlays particularly in plates inoculated with high levels of  $\phi$ C2(W).



## DISCUSSION

The objectives of this study were to partially characterise a previously un-reported phage for *Str. lactis* C2 and to attempt to elucidate why paired or multi-strain cultures infected with this phage exhibited reduced acid production although the non-homologous strains did not propagate the phage.

Since  $\phi$ C2(W) has not been described previously, partial characterisation of the phage was undertaken to enable the differentiation of this phage from other phages for C2 or the related strains ML3 and 712. The latent period was unusually short for a lactic streptococcal phage. Values ranging from 23-56 min at 30°C have been reported (Keogh, 1973; Pearce and Lawrie, 1974). However, these values were obtained in RSM and different results would probably have been found if the determinations were performed in another medium. The short latent period combined with its fairly large burst size explains the rapid multiplication of this phage found in milk and broth media.

Relatively few accounts of halo production by lactic streptococcal phages exist (Pette, 1953; Naylor and Czulak, 1956; Tourville and Johnstone, 1966; Douglas, Qanber-Agha and Phillips, 1974; Terzaghi and Sandine, 1975). Halo production is indicative of the production of either capsule degrading enzymes (polysaccharide depolymerases) or cell wall degrading enzymes (Tsugita, 1971). A calcium requirement for halo formation has not been previously described for this group. The effect of calcium can be explained if  $\phi$ C2(W) induces C2 to produce a cell wall degrading enzyme. Such enzymes have been shown to be activated by cations (Oram and Reiter, 1965; Tourville and Tokuda, 1967).

On the basis of replication response to temperature  $\phi C2(W)$  gave a group 1 response as defined by Mullan *et al.* (1981b) and was similar in this respect to the C2 phage studied by these workers. Contrary to the findings of Archibald *et al.* (1978) the growth rate of C2 was not the major constraint on the rate of replication of  $\phi C2(W)$ ;  $\phi C2(W)$  replicates faster at 38°C and 40°C than at 30°C whereas the C2 host had a faster growth rate at 38°C than at 30° or 40°C.

Morphologically  $\phi C2(W)$  may be classified within Bradley's group B (Bradley, 1967) and is similar in appearance to another C2 phage described by Keogh and Shimmin (1974). Their phage, however, differed slightly in dimensions and possessed a collar. The  $\phi ML3$  phage studied by Oram and Reiter (1965) is also similar morphologically to  $\phi C2(W)$  (see Table 1.20) but differs slightly in dimensions. The  $\phi C2(W)$  virus also differs from  $\phi ML3$  in other respects;  $\phi ML3$  demonstrates lysis-from-without whereas  $\phi C2(W)$  does not and the lytic enzyme induced by  $\phi C2(W)$  differs in several characteristics from  $\phi ML3$  lysin (see Chapter 2).

Preparations of lactic streptococcal phages are generally concentrated and partially purified by high speed centrifugation using density gradients. Lawrence *et al.* (1976) have commented that electron micrographs of these viruses have shown many broken or empty headed particles which may be indicative of inadequate sample preparation. Davies (Dr. L. Davies, National Institute for Research in Dairying, pers. comm.) has found that the above procedure frequently results in large clumps of phage which create difficulties in quantitative studies of phage morphology. The electron micrographs of  $\phi C2(W)$  obtained in this study did not show significant clumps of phage and revealed that the vast majority of phage had intact heads which also contained DNA. The use of ultrafiltration to concentrate and purify lactic streptococcal bacteriophages, possibly combined with negative chromatography

on the dye ligand Green A, may be of value in the study of phage morphology.

Lysates of  $\phi$ C2(W) were shown to contain a lytic agent which lysed both homologous and non-homologous strains. The lytic agent was clearly differentiated from particulate phage on the basis of heat sensitivity, chromatography on the dye ligand Green A, solubility in acetone, and particle weight. The pH and heat sensitivity of the lytic agent are consistent with the agent being an enzyme. The finding that the rate of cell lysis was directly proportional to the concentration of lytic agent also suggested that lysis was due to a cell wall degrading enzyme.

The production of lysin by *Str. lactis* C2 was shown to be phage induced. Lysin was not detected in un-infected cells of C2 or in cells infected with  $\phi$ 712. The available data suggests that lysin production represents new enzyme formation and that the information for lysin production is contained in the  $\phi$ C2(W) genome. From the kinetics of lysin production it seems probable that lysin is involved in the release of  $\phi$ C2(W) from infected cells.

Evidence that  $\phi$ C2(W) lysin was involved in the entry of viral DNA into host cells was not found. The failure to liberate lytic activity from high titre preparations of  $\phi$ C2(W) coupled with the inability of this phage to cause lysis-from-without suggests that this virus does not possess a lytic enzyme associated with structural components. Additional evidence that lysin is not involved in DNA introduction can be inferred from the difference in heat stability between phage and lysin. If lysin were involved, a drastic drop in the infectivity (titre) of  $\phi$ C2(W) particles would be expected after 1 or 5 min at 57°C (see Table 1.12). Since both these treatments eliminate lytic activity but not phage it is unlikely that

$\phi$ C2(W)-induced lysin is involved in infection of C2 by  $\phi$ C2(W). Perhaps the involvement of lysin in phage release is the explanation for the failure of the author to isolate 'haloless' mutants of  $\phi$ C2(W); such mutants may be unable to form plaques because insufficient lysin is produced to allow the exit of progeny phages required for the initiation of the cycles of infection and multiplication required for plaque formation. The frequency at which such mutations occur may also be too low to permit the easy isolation of a lysin-deficient mutant.

The  $\phi$ C2(W)-C2 system is not unique in the sense that the phage induces the host to produce a lytic enzyme although the phage itself does not possess a lytic enzyme. Ralston *et al.* (1961) studied virolysin production by phage-infected staphylococci but reported no evidence for a lytic enzyme associated with phage particles. Similarly, Goepfert and Naylor (1967) failed to demonstrate lytic activity with a phage for *Micrococcus lysodeikticus* although this virus also induced the production of a lytic enzyme by its host. It has been suggested by Emrich and Streisinger (1968) that the lytic enzyme associated with some phage particles is not an integral structural component but is non-specifically adsorbed to various phage components (DNA or protein). If this is so then medium composition, particularly the ionic composition, and the basicity of the lysin may be factors which influence whether or not lysin is associated with phage particles and may explain why certain phages apparently have a lytic enzyme.

Although several authors (Reiter and Oram, 1965; Reiter, 1973; Marshall and Berridge, 1976; Law and Sharpe, 1978) have referred to the role of phage tail lysin in the digestion of the cell wall in lactic streptococci, to permit DNA introduction, definitive proof of lysin involvement in this process has yet to be demonstrated. Emrich and

Streisinger (1968) questioned the role of T2 phage lysozyme in penetration since a lysozyme-deficient mutant of T2 was as infective as the wild type phage.

How then does  $\phi C2(W)$  infect C2? Unlike phage for many other bacterial groups, some lactic streptococcal phages have receptor sites, not on the cell wall, but on the plasma membrane (Oram and Reiter, 1968; Oram, 1971). If holes exist in the cell wall of C2, which are filled with protrusions of the plasma membrane, as was found for a *Str. lactis* strain by Hurst and Stubbs (1969) then direct phage membrane contact could take place. The holes found by the previous workers are sufficiently large (20 nm), if they exist, to permit entry of the tail of  $\phi C2(W)$  and allow contact with the plasma membrane. Tentative evidence for close association of membrane and wall in *Str. lactis* may be inferred from the difficulty experienced by various workers in obtaining cell walls free of lipid contamination (White and Hurst, 1968; Oram, 1971). However, Lawrence *et al.* (1976) have questioned the interpretation of the 'holes' in the electron-micrographs published by Hurst and Stubbs (1969) and the possibility therefore exists that they are artifacts. Obviously further work is required to elucidate the mechanism(s) involved in the infection of Group N streptococci by phage.

Comparison of  $\phi C2(W)$  with other phages which also induce lysin production is difficult because of lack of published data. In addition the data available must be interpreted with caution because they may have been obtained by a worker other than the researcher who performed the studies on lysin production. Also, the experiments may have been undertaken many years further on from the lysin studies with the risks of the occurrence of phage mutation, contamination of virus preparations with other phages, and/or mislabelling of phage preparations. Data are available

for  $\phi$ C10-1,  $\phi$ C10-11 and  $\phi$ ML3 and is presented in Table 1.20. With the exception of morphology it is difficult to see any relationships between the four lysin-producing phages. All are of the prolate polyhedral type but differ with respect to dimensions and the possession of base plates.

In marked contrast both  $\phi$ C10-111 and  $\phi$ 712 have a completely different morphology. This study has revealed that  $\phi$ 712 has an isometric head. This finding has been confirmed by Davies (pers. comm.).  $\phi$ C10-111 also has an isometric head (Keogh and Shimmin, 1974).

It may be significant that all four lysin-producing phages are of the prolate type. Prolate type phages have been found to occur at low frequency in whey in New Zealand. In a survey over the period 1974-1977, 89% of the phages studied were isometric, the remaining 11% were of prolate morphology (Lawrence *et al.*, 1978). It would be interesting to survey phages of different morphological groups for lysin production to determine if relationships exist between morphology group and lysin production. If it can be demonstrated that only prolate phages produce significant concentrations of lysin, the lower incidence of this morphological group cannot be used as evidence for complacency since prolate phages have a much wider host range than isometric phages (Lawrence *et al.*, 1978).

The nascent effects of  $\phi$ C2(W) were demonstrated against paired and multi-strain cultures containing C2. Evidence was presented that the inhibition of acid production experienced when cultures containing C2 and a non-homologous strain were infected with  $\phi$ C2(W) was due to the lysis of the non-homologous strain by lysin produced by phage-infected cells of C2. These results confirm the findings of Naylor and Czulak (1956) that phage-induced lysins can give rise to the nascent phage phenomenon. The involvement of lysins in the nascent phenomena has also been suggested by Reiter and Oram (1963), Oram and Reiter (1965), Tourville and Johnstone

Table 1.20

Some properties of lysin producing lactic streptococcal phages

Bacteriophage	Halo production	Latent period	Burst size	Type of head	Head dimensions (nm)	Tail dimensions (nm)	Presence of collar baseplate	hosts	Heat treatment required for lysin destruction	Reference
φC10-1	+	48 min <sup>(1)</sup>	40 <sup>(1)</sup>	Prolate polyhedral <sup>(2)</sup>	51 x 54 <sup>(2)</sup>	92 x 5 <sup>(2)</sup>	-	C10	60°C, 5 min	Naylor and Czulak (1956)
φC10-2	+	52 min <sup>(1)</sup>	27 <sup>(1)</sup>	Prolate polyhedral <sup>(2)</sup>	55 x 42 <sup>(2)</sup>	92 x 9 <sup>(2)</sup>	-	C10	60°C, 5 min	Naylor and Czulak (1956)
φML3	+			Prolate polyhedral <sup>(3)</sup>	55 x 40 <sup>(3)</sup>	100 x 9 <sup>(3)</sup>	-	ML3, 712	Rapid inactivation at temperatures >37°C	Gram and Reiter (1965)
φC2(W)	+	20 min	148	Prolate polyhedral	44-46 x 54-56	90 x 8.5	-	C2, 712, ML3		PS

<sup>(1)</sup> Determined by Keogh (1973) in R.S.M.<sup>(2)</sup> Determined by Keogh and Shimmin (1974).<sup>(3)</sup> Determined by Bradley and Kay (1960).

PS Present study.

(1966) and Reiter (1973). No evidence for a direct role for phage as suggested by Collins (1952a), Whitehead *et al.* (1952) and Whitehead *et al.* (1953) was found.

Two cultures AM2 + C2 and US3 + C2 did not demonstrate nascent effects when infected with  $\phi$ C2(W). Detailed study of the component strains with purified lysin revealed that US3 and AM2 were relatively resistant to phage lysin (Chapter 2). This finding lends additional support to the involvement of lysin in the nascent phenomenon.

Although Pette (1953) showed that phage lysins had a broader lytic spectrum than phage, an observation which has been confirmed by Naylor and Czulak (1956), Oram and Reiter (1965) and Tourville and Tokuda (1967) lysin-producing phages and the effects of infecting paired and multi-strain cultures with such phages appear to have received little, if any, attention. The results of the present study show that infection of paired and multi-strain cultures with a lysin-producing phage can result in significant inhibition of acid production.

For about 40 years, cultures containing two or more phage-unrelated strains have been used as part of phage control schemes in dairy fermentation plants. The rationale for their use depends on the ability of phage-resistant strains to continue to produce acid when one or more strains are infected with homologous phage. It is apparent from the literature (Pearce *et al.*, 1970; Christensen, 1972; Lawrence *et al.*, 1976; Lawrence *et al.*, 1978; Muskowitz *et al.*, 1979; Walker *et al.*, 1981) that this method of phage control has not been completely successful. The results of this study have clearly shown a major deficiency in the use of paired or multi-strain cultures in phage control schemes and provide one explanation for the lack of success of such starters in eliminating the



problem of phage-induced slow acid production in cheese factories; if one of the component strains is attacked by a lysin-producing phage and the other phage-unrelated strain or strains is/are sensitive to lysin, it is probable that acid production will be inhibited because the lysin produced by the phage-infected strain will destroy the phage resistant strains.

Because nascent type effects can occur when the propagating host for the lysin producing phage constitutes only a small proportion of the initial starter population it is tempting to speculate that there are incidents of slow acid production in cheese factories due to the nascent phage phenomenon which have not been attributed to phage. The results of this study have shown that nascent effects can be demonstrated with as little as 5% host in a multiple-strain culture whereas phage detection using the double agar assay method required the presence of about 20% host for the lysin producing phage. That nascent effects can occur when the host comprises only a small proportion of the initial cell population was first reported by Evans (1934) and is confirmed by the results of this study.

The  $\phi$ C2(W)-C2 system may be unusual in that plaques were demonstrated using only 20% (v/v) of host in the inoculum for the double agar assay method. In many other lactic streptococcal phage-host systems a requirement of at least 33% homologous host has been demonstrated (Mullan, unpublished information; Dr. G. Stanley, Unigate Central Laboratory, pers. comm.).

If it can be shown that  $\phi$ C2(W) and other lysin producing phages are not atypical (i.e. super lysin producers) and constitute a significant part of the phage flora of dairy fermentation plants, then the susceptibility of strains to phages which direct the host-synthesis of high concentrations

of lysin and the sensitivity of strains to lysin may be two additional criteria which require consideration in the selection of lactic streptococci for inclusion in starter cultures.

The preliminary report of Jarvis (1980) is interesting since it states that 30 out of 52 phage lysates tested produced a lytic effect (due to lysin) on at least one out of 39 strains and that 29 out of 39 strains of lactic streptococci were sensitive to at least one phage lysin and suggests that lysin-producing phages are a normal part of the phage flora of cheese factories.

The advantages of strain selection are apparent from the data obtained for infected cultures of C2 + AM2 and C2 + US3 which exhibited little inhibition of acid production when infected with  $\phi$ C2(W). Detailed studies with AM2 and US3 and purified phage lysin (see Chapter 2) revealed that these strains were relatively insensitive to lysin. Obviously strains resistant or relatively resistant to lysin exist or can be obtained by mutation which shows that the compilation of cultures containing strains insensitive to phage lysin is feasible and could provide a method for preventing inhibition of acid production by lysin-producing phages.

Lysin production by phage-infected lactic streptococci may not be detrimental in all instances. That phage-carrying (the pseudo-lysogenic condition) lactic streptococci are more phage-resistant than non-phage carrying strains has been established (Hunter, 1947; Limsowtin and Terzaghi, 1977). Barksdale and Arden (1974) recognised three mechanisms of phage-host interaction whereby the carrier state could arise. In one mechanism, phage-infected cells produce enough phage lysin to destroy the phage receptor sites and artificially render most of the population phage resistant. However, the mechanisms whereby the carrier state is achieved in the lactic streptococci have not yet been elucidated. Since the phage

resistance enjoyed by mixed-strain starters has been attributed, at least in part, to the existence of the carrier state within these cultures (Hunter, 1947; Crawford and Galloway, 1962; Galesloot, Hassing and Stadhouders, 1966) phage lysins may have the potential to have both detrimental and beneficial effects on starter performance during cheese-making and other dairy fermentations.

### SUMMARY

A phage for *Str. lactis* C2 which induces nascent effects was studied and an attempt was made to elucidate the mechanism responsible for the nascent phenomenon.

Supplementation of assay media <sup>with Ca<sup>++</sup></sup> was not required for ~~plaque~~ formation but was necessary for the demonstration of zones of secondary lysis (haloes) around plaques.  $\phi$ C2(W) had an unusually short latent period about 21 min, and lysed *Str. lactis* strains C2, 712 and ML3. The phage had a prolate head and a non-contractile tail and could be classified within Group B of Bradley's morphological classification scheme. Phage replication occurred at 22°, 30°, 38° and 40°C and on the basis of replication response to temperature could be classified with Group 1 of Mullan *et al.* (1981b).

Infection of eight paired strain cultures, each containing *Str. lactis* C2 and a phage-unrelated strain, with  $\phi$ C2(W) resulted in marked inhibition of acid production for six cultures. Since adsorption of  $\phi$ C2(W) did not occur or occurred to only a low extent for some of the non-homologous component strains a direct involvement of  $\phi$ C2(W) in the inhibitory process appeared unlikely.

Lysates of  $\phi$ C2(W) were shown to contain a lytic agent which lysed phage resistant as well as phage-sensitive lactic streptococci. An assay method for measuring lytic activity was developed using freeze-dried cells suspended in 0.1 M potassium phosphate buffer, pH 6.7 and is described

Lytic activity in  $\phi$ C2(W) lysates was clearly shown to be distinct from that of particulate phage. Phage levels could be reduced by 99% by centrifugation or by 95% by acetone precipitation without significantly affecting the level of lytic activity in broth lysates. Lytic activity

but not phage was destroyed by mild heat treatment and was lost after ultrafiltration or chromatography on the dye ligand Green A.

Attempts to demonstrate lytic activity due to lytic enzymes associated with the  $\phi$ C2(W) particle were unsuccessful. Infection of C2 cells with a phage-coccus ratio of 10,000:1 failed to give mass lysis. Attempts to yield a lytic enzyme from concentrated preparations of  $\phi$ C2(W) by sonication were also unsuccessful and yielded further evidence that the lytic agent present in  $\phi$ C2(W) lysates was not phage.

It was shown that lytic activity was phage-induced. Lytic activity was demonstrated in cells of C2 infected with  $\phi$ C2(W) 10 min after infection; about half way through the latent period. The concentration of lytic agent increased thereafter reaching high concentrations at the rise period. Uninfected cells of C2 did not contain lytic activity. Infection of C2 with  $\phi$ 712 did not result in lysates of significant lytic activity.

Experiments designed to investigate the mechanism involved in the inhibition of acid production experienced when cultures containing C2 were infected with  $\phi$ C2(W) yielded evidence that the inhibition was due to the lysis of the non-homologous strain by phage-induced lysin. Inhibition of acid production was not observed when cultures were infected with  $\phi$ 712. This phage does not induce lysin production. Nascent effects did not occur when cultures containing C2 and lysin and phage resistant strains were infected with  $\phi$ C2(W). Lysis of paired strain cultures infected with  $\phi$ C2(W) was demonstrated using spectrophotometric methods. Similar experiments with  $\phi$ 712 did not give mass lysis.

Nascent effects were also demonstrated using multi-strain cultures and in multi-strain cultures containing only 5% *Str. lactis* C2. However, plaque formation by  $\phi$ C2(W) required the presence of about 20% C2 in cultures.

The significance of these results to starter selection for cheese-making and possible control measures are discussed.

## CHAPTER 2

ISOLATION, PARTIAL PURIFICATION AND  
CHARACTERISATION OF THE BACTERIOLYTIC AGENT  
PRODUCED BY THE  $\phi$ C2(W)-C2 PHAGE-HOST SYSTEM

## INTRODUCTION

The presence of phage-induced cell wall or capsule dissolving (lytic) enzymes in lysates of phage-infected bacteria has been described for *Escherichia coli*, *Staphylococcus aureus*, *Azotobacter agilis*, *Aerobacter cloacae*, *Bacillus megaterium*, *Micrococcus lysodeikticus*, *Bacillus stearothermophilus*, *Klebsiella pneumonia*, *Pseudomonas putida*, *Ps. aeruginosa* and for streptococcal phage-host systems (for a review see Tsugita, 1971). Lysis of lactic streptococci by phage lysins was first reported by Pette (1953). Other reports of lysin production by lactic streptococcal bacteriophages have been presented by Naylor and Czulak (1956), Reiter and Oram (1963), Oram and Reiter (1965), Tourville and Johnstone (1966) and Tourville and Tokuda (1967).

Pette (1953) established that phage lysins had a broader lytic range than phage. Naylor and Czulak (1956) confirmed this finding and also found that lysins were more heat labile than phage. Oram and Reiter (1965) and Reiter and Oram (1963) reported the partial purification of a lytic enzyme from phage ( $\phi$ ML3) lysates of *Str. lactis* ML3. Their method involved acetone precipitation (to remove phage), ion exchange chromatography and salt precipitation. The ~~phage~~ enzyme was purified about 500-fold and contained about  $7.5 \times 10^7$  pfu/ml. Unlike some other phage associated lysins which only lysed damaged cells or viable cells sensitised by phage (e.g. Ralson *et al.*, 1957; Murphy, 1961) the  $\phi$ ML3 enzyme lysed viable cells of all strains of lactic streptococci tested. Strain dependent differences in sensitivity were noted, however. Maximum lytic activity was found at pH 6.6-6.9. The enzyme was activated by monovalent cations and to a lesser extent by divalent cations. From an examination of cell wall degradation products the authors concluded that the lytic enzyme was a glycosidase with a specificity similar to hen egg lysozyme, i.e.



$\phi$ ML3 lysin is an N-acetylmuramidase. Most enzymes have a temperature coefficient ( $Q_{10}$ ) of about 2 (Dixon and Webb, 1964) but the phage enzyme was unusual in that it had both a high  $Q_{10}$ , about 4.9, and a high activation energy, about 28.0 kcal mole<sup>-1</sup>. The lysin was inhibited by sulphydryl reagents, which suggested that a sulphydryl group was essential for activity. Lytic activity was reported to be phage-induced and was more heat-labile than phage.

Tourville and Tokuda (1967) purified a lytic enzyme from a  $\phi$ C10 lysate of *Str. lactis* C10. Purification was achieved by a multi-step procedure involving ultracentrifugation (to remove phage), salt precipitation, gel filtration and ion exchange chromatography. The lytic enzyme was purified about 1,700-fold and contained about  $10^4$  pfu/ml. The  $\phi$ C10 enzyme was activated by monovalent and divalent cations and had a high temperature coefficient (3.9) and activation energy. The enzyme was highly basic and had an isoelectric point in excess of 8.6. The sedimentation rate of  $\phi$ C10 lysin was reported to be about 7S based on sucrose gradient studies using albumin, 7S and 19S antibodies. This figure is compatible with a molecular weight for the lysin in excess of 200,000 daltons. Lytic activity was inhibited by sulphydryl reagents and maximum lytic activity was obtained at pH values between 6.0 and 6.5. The heat lability of the lysin was confirmed.

In this section attempts to purify and characterise the lytic agent present in  $\phi$ C2(W) lysates are presented.

## MATERIALS AND METHODS

### ORIGINS OF CULTURES AND BACTERIOPHAGES

Cultures with the prefix NCD0 before the strain identification code were obtained from the National Collection of Dairying Organisms, National Institute for Research in Dairying, Reading, England. Strains of *Str. thermophilus*, *Str. cremoris* strains SK11, R6 and *Micrococcus lysodeikticus* were also obtained from this source. *Str. lactis* strains RM1, C2, C10, ML3 and *Str. cremoris* strains HP and E8 were obtained from the culture collection maintained at this Department. Cultures of *E. coli*, *Lactobacillus bulgaricus* and *Lact. fermenti* were also obtained from the Department's collection. *Str. lactis* ML8 and *Str. cremoris* strains US3 and AM2 were obtained from the culture collection of Dr. T. Cogan, Agricultural Institute, Fermoy, County Cork, Ireland. *Str. diacetylactis* DRC2 was obtained from Dr. B. Keogh, C.S.I.R.O., Melbourne, Australia. *Str. cremoris* 1249 and *Str. diacetylactis* 1246 were obtained from Dr. G. Stanley, Unigate Technical Centre, Trowbridge, England. *Str. cremoris* SC1 was kindly supplied by Dr. W. E. Sandine, Oregon State University, U.S.A. The origin of  $\phi$ C2(W) has been previously described (p20).

### CULTURE MAINTENANCE

Cultures of Group N streptococci were maintained as described previously (p20). Group D streptococci were maintained as for the Group N streptococci except that strains were incubated at 37°C. The *Str. thermophilus*, *Lactobacillus* and *Leuconostoc* strains were grown in MRS broth (De Man, Rogosa and Sharpe, 1960). The *Str. thermophilus* and *Lactobacillus* strains were propagated at 37°C using a 1% inoculum and an incubation period of 14-16 h. The *Leuconostocs* were incubated using a temperature of 30°C, an inoculum level of 1% and an incubation time

of 16-24 h. Strains of *E. coli* were maintained on nutrient agar (Oxoid) slopes. Slopes were inoculated with loopfuls of an active *E. coli* strain in nutrient broth and incubated at 37°C for 24 h. Slopes were stored at 5°C until required. *M. lysodeikticus* was propagated in PLGYG broth using similar conditions to those for Group N streptococci. Between transfers all cultures were held at 5°C. For longer term storage cultures, with the exception of *E. coli*, were inoculated into the appropriate growth medium using a 3% (v/v) inoculum and immediately frozen at -30°C.

#### DETERMINATION OF PROTEIN AND SPECIFIC ACTIVITY OF $\phi$ C2(W) LYSIN

Protein was measured using the Bio Rad assay described previously (p29). Protein and lysin assays were used to monitor the purification of phage lysin during ion-exchange chromatography, salt precipitation, ultrafiltration and gel filtration. The values obtained were used to calculate the specific activity of the lysin at each stage of enzyme purification. Specific activity, which is the ratio of some measure of enzyme concentration e.g. activity, to the total protein concentration, usually in mg/ml, should increase during purification. The degree of purification at a particular stage may be obtained by dividing the specific activity at that point by the specific activity of the starting material. The value of specific activity for this purpose has been discussed by Dixon and Webb (1964). In this study specific activity was determined using the formula:

$$\frac{\text{lysin activity (u/ml)}}{\text{protein concentration (\mu g/ml)}}$$

#### PRODUCTION OF BROTH LYSATES CONTAINING HIGH LEVELS OF LYTIC ACTIVITY

Preliminary experiments (Table 1.8) established that there was a relationship between the phage and lysin levels in a  $\phi$ C2(W) lysate; the higher the phage level the higher the concentration of phage lysin. The following procedure was used in all small scale experiments concerned

with lysin isolation and purification to produce broth lysates containing high levels of lytic activity. Broth media were inoculated with 2% (v/v) of C2 and grown to an O.D.<sub>450</sub> of about 0.58-0.6 at 30°C. Sufficient 1 M calcium chloride was then added to give an added concentration of 10 mM Ca<sup>++</sup> and the culture was infected with  $\phi$ C2(W) to give a phage : coccus ratio of about 10. After incubation for a further 2½ h at 30°C lysates were obtained which contained 120-180 u/ml of lytic activity. Unless stated otherwise PLGYG broth was used for lysin production. In some experiments other media, designated TYGG and TLBC were used. TYGG contained (g/l) tryptone (Oxoid), 5; yeast extract (Oxoid), 2.5; glucose, 5 and  $\beta$ -disodium glycerophosphate (Sigma - grade II), 7.2. TLBC contained (g/l) tryptone (Oxoid), 5; lab lemco (Oxoid), 5; glucose, 5; glycerophosphate (Sigma - grade II), 7.2.

#### ION EXCHANGE CHROMATOGRAPHY

Phage-induced lysins are basic proteins. In an acidic environment the ionisable amino groups of such proteins acquire protons and the molecule thus acquires positively charged  $-\text{NH}_3^+$  groups. Under suitable conditions  $-\text{NH}_3^+$  groups can be bound by cation exchange resins and protein isolation and, ultimately, purification is achieved. In practice, weak carboxylic resins containing carboxylic acid exchange groups on a micro-reticular acrylic polymer have found wide application in this field of enzyme purification because basic enzymes can be eluted under 'mild' conditions (e.g. increasing pH and/or ionic strength of eluant buffer) which avoid denaturation. In this project weak carboxylic acid ion exchange resins were used to isolate, concentrate and partially purify  $\phi$ C2(W)-induced lysin.

## Resins

Amberlite CG50 (100-200 mesh) was obtained from Sigma Chemicals, Poole, Dorset, England. Bio Rex 70 (20-50 mesh) was obtained from Bio-Rad Laboratories. The latter product was essentially a highly purified version of the former. The main difference between the two resins was particle size.

## Preliminary experiments with CG50

In these experiments the lytic agent present in  $\phi$ C2(W) lysates was isolated using CG50 essentially as described by Oram and Reiter (1965). An excess of resin was hydrated in an excess of distilled water and fines removed by decantation. The pH of the resin was adjusted to 5.5 with dilute hydrochloric acid and the slurry poured into a 45 cm x 1.5 cm column, partially filled with distilled water. The resin was packed to a bed height of 30 cm and equilibrated by passing four bed volumes of 60 + 40 mixture of 0.002 M potassium phosphate (pH 5.5) + acetone respectively through the column (Oram and Reiter, 1965). Phage lysates, after partial removal of  $\phi$ C2(W) by acetone precipitation (p26), were adjusted to pH 5.8 and pumped through the column with a Watson and Marlow variable speed peristaltic pump (Watson-Marlow, Falmouth, Cornwall, England) type MHRE 200 to give a flow rate of  $17 \text{ ml min}^{-1}$ . The column was washed with about ten bed volumes of 0.002 M phosphate buffer (pH 5.5) and elution initiated. Lytic activity was eluted with 0.2 M potassium phosphate buffer pH 7.3 and 10 ml fractions were collected using a Model 1200 fraction collector, Instrumentation Specialities Company, 4700 Superior, Lincoln, Nebraska 68504, U.S.A. (ISCO fraction collector). Experiments were performed at 5°C.

## LARGE SCALE ISOLATION AND PARTIAL PURIFICATION OF $\phi$ C2(W)-INDUCED LYSIN

### Preparation of $\phi$ C2(W) lysates

TYGG broth was used to prepare  $\phi$ C2(W) lysates. The medium (108L) was inoculated with 3% (v/v) of a late stationary phase culture of C2 and incubated at 30°C until the O.D.<sub>450</sub> reached 0.6. Sufficient sterile sodium hydroxide (20% w/v) was added to bring the pH to about 7 and the medium was supplemented with 10 mM Ca<sup>++</sup>. Sufficient phage was then added to give a concentration of about 10<sup>6</sup> pfu/ml. Incubation was continued for a further 2 h at which time the O.D.<sub>450</sub> had dropped to 0.21. Chloroform (200 ml) was then added to prevent further bacterial growth and the lysate was distributed in 45  $\ell$  lots and held at 5°C overnight. Next day (24 h) the lysate was pumped (35  $\ell$  h<sup>-1</sup>) through a Westfalia self desludging centrifuge model no. SA00H to remove unlysed cells and cellular debris. This process reduced the O.D.<sub>450</sub> from 0.21 to 0.11.

### Resin preparation

Amberlite CG50 (100-200 wet mesh) was freed from fines by decanting with distilled water (about 18 decantations) converted to the H<sup>+</sup> form by treatment with dilute phosphoric acid and adjusted to pH 6.2 with potassium hydroxide. The resin was then used to pack a 90 cm x 50 cm column. The column was equilibrated by pumping 12  $\ell$  of 0.09 M potassium phosphate buffer, pH 6.2 through at a flow rate of 15  $\ell$  h<sup>-1</sup>. The bed height was adjusted to 44 cm and the void volume was about 1600 ml.

### Isolation and concentration of $\phi$ C2(W) lysin

The pH of the lysate was adjusted to 6.2 and <sup>the lysate</sup> was pumped through the column at a flow rate of 10-15  $\ell$  h<sup>-1</sup>. After the lysate had been

applied, the column was washed thoroughly with 0.09 M potassium phosphate buffer pH 6.2 until the eluate pH reached 6.1-6.2. At this time elution was initiated with 0.2 M potassium <sup>phosphate</sup> buffer pH 7.3. The first 2  $\ell$  were discarded and 200 ml fractions collected using a Pharmacia preparative fraction collector PF-30 (Pharmacia (Great Britain) Ltd., Prince Regent Road, Hounslow, Middlesex). Little enzyme was found in the first 6.5  $\ell$  effluent but was present in the following 10  $\ell$ . Fractions containing high levels of lytic activity were pooled and analysed for protein, phage and lytic activity using methods described previously. Pooled fractions were divided into 2.5  $\ell$  lots and frozen at  $-30^{\circ}\text{C}$  until required for further purification or other studies. Little loss of enzyme activity occurred in samples stored under these conditions, over a 2½ year period.

#### GEL FILTRATION STUDIES

Gel filtration was used in an attempt to fractionate the lytic activity of crude and partially purified  $\phi\text{C2(W)}$  lysates, in enzyme purification, and to determine the molecular weight of  $\phi\text{C2(W)}$ -induced lysin.

Gel filtration is a liquid chromatographic method which separates molecules primarily according to differences in molecular dimensions. In this method particles of a gel material with a sponge-like matrix structure, containing pores of controlled dimensions, are poured into a column under conditions which permit uniform packing of the particles. A sample which contains a mixture of substances differing in molecular dimensions is then applied to the bed surface and subsequently caused to percolate through the bed by eluant flow. As elution proceeds, molecules which are too large to enter the pores of the gel matrix pass rapidly through the bed in the space surrounding the gel particles, and are eluted in a single zone near the beginning of the elution profile. On the

other hand, molecules which are capable of diffusing into the pores of the matrix are retarded in their migration through the bed. The extent of retardation is inversely correlated with molecular dimensions; therefore the smallest molecules are retarded to the greatest extent and are thus the last to emerge from the bed.

#### Gel filtration media

In initial experiments and in molecular weight determination studies polyacrylamide gel filtration media were used. These hydrophilic gel media are produced by the co-polymerisation of acrylamide with the cross-linking agent N,N' methylene-bis-acrylamide and differ from sephadex type media in that they contain only low concentrations of anionic groups (Pecsok and Saunders, 1968; Anon., 1975). This property should be particularly valuable in studies with phage lysins. Phage lysins are very basic proteins and the use of chromatographic media with appreciable cation exchange capacity could result in significant enzyme losses. In later experiments Sephacryl S-200 was used. This is a relatively new gel filtration medium and is produced by covalently cross-linking allyl dextran with N,N methylene bisacrylamide.

#### Preparation of gel filtration media

Bio-Gel P100 (50-100 mesh) and Bio-Gel P150 (50-100 mesh) were obtained from Bio-Rad Laboratories Ltd. Sephacryl S200 was obtained from Pharmacia. Bio-Gel media were hydrated in an excess of elution buffer for 24 h at room temperature essentially as described in the Bio-Rad Gel Chromatography Manual (Anon., 1975). During this period, and after hydration, fines were removed by suction until no turbidity remained in the supernatant. Exhaustive fines removal was essential to obtain



a satisfactory flow rate with Bio-Gel P150. Sephacryl was supplied swollen and was equilibrated in elution buffer prior to packing.

#### Column packing

Gel slurries in the respective elution buffer were degassed under vacuum, cooled to 5°C and carefully poured into a Pharmacia K26/100 column (2.6 cm x 100 cm) which contained about 10 ml of elution buffer. Prior to pouring the slurry, eluant was injected into the outlet tubing and pumped up through the bed support to remove air bubbles. A column extension (Pharmacia product R25/26, capacity 500 ml) was used and enabled columns to be packed in one operation, thus ensuring even sedimentation of the gel particles. With the Bio-Gel media, particles were allowed to sediment for 30 min at which time eluant flow was initiated. After sedimentation for 10 min flow was initiated with columns containing Sephacryl.

Bio-Gel P150 and Bio-Gel P100 were packed using a hydrostatic head of 41 cm and 51 cm respectively. After equilibration with eluant this gave flow rates of 10 ml h<sup>-1</sup> and 60 ml h<sup>-1</sup> respectively. Sephacryl was packed by pumping elution buffer at a flow rate of 180 ml h<sup>-1</sup> through columns. A varioperpex pump (LKB, Produkter AB, S-161.25 Brommöl, Sweden) was used in all gel filtration experiments in which pump elution was used. Flow was varied by altering the pump speed.

In preliminary experiments columns were operated in the descending mode and the flow rate was varied by altering the hydrostatic head. A constant head was maintained using a 500 ml Mariotte flask. Later experiments used pump elution and ascending flow. Columns containing P100, P150 and Sephacryl S-200 were eluted with 0.1 M potassium phosphate buffer pH 7.0, 0.2 M potassium phosphate buffer pH 7.3 containing 0.01% (w/v) sodium azide, and 0.1 M potassium phosphate, pH 7.0, containing 5 mM

ethylenediaminetetracetic acid (EDTA), 5 mM mercaptoethanol and 0.01% (w/v) sodium azide respectively unless stated otherwise. All buffers were filtered through Whatman No. 5 filter paper to remove particulate contaminants and were degassed by aspirating solutions under vacuum.

#### DETERMINATION OF THE MOLECULAR WEIGHT OF $\phi$ C2(W)-INDUCED LYSIN

A Pharmacia K26/100 column was packed with Bio-Gel P100 (50-100 mesh) equilibrated with 0.2 M potassium phosphate buffer, pH 7.3, containing 0.01% (w/v) sodium azide to a bed height of 90 cm as previously described. The column was operated at 5°C and was eluted in the descending mode by pump elution with the equilibration buffer. A higher ionic strength and pH were used in this experiment than in other gel filtration experiments to minimise the possibility of lysin-gel matrix interactions.

#### Protein standards

Columns were calibrated using the following proteins: bovine serum albumin, molecular weight 67,000; ovalbumin, molecular weight 43,000; chymotrypsinogen A, molecular weight 25,000; and Ribonuclease A, molecular weight 13,700. Blue dextran 2000, molecular weight  $2 \times 10^6$ , was used to determine the void volume. The calibration proteins and blue dextran were obtained from Pharmacia. In calibration experiments 10 mg of each protein, except ribonuclease A of which 13 mg were used, were dissolved in 5.0 ml of elution buffer and 4.5 ml of the mixture used in gel filtration experiments. To avoid the possibility of protein-protein interactions creating erroneous results several proteins were also run separately.

#### Sample application

The column end piece was removed and the bed surface, which was protected by a sample application cup, drained. The calibration protein

mixture (4.5 ml) was carefully added and allowed to drain into the gel. Samples were washed into the gel with 2.0 ml of elution buffer. After the sample had been washed in, the column was refilled with eluant and elution started.

#### Fraction collection

Effluent was collected using the model 1200 ISCO fraction collector. The collector was operated in the time mode and fractions were collected at predetermined intervals. The absorbance of fractions at 280 nm was determined using a Pye Unicam SP-1800 double beam spectrophotometer (Pye Unicam Ltd., York Street, Cambridge, England) and the peak elution volume ( $V_e$ ) for each protein standard obtained.  $V_e$  values were plotted against the logarithm of the molecular weight of the protein standard as described by Andrews (1964) to prepare  $V_e/\log$  molecular weight calibration curves. The molecular weight of the  $\phi C2(W)$  lysin was obtained by applying crude or partially purified preparations of  $\phi C2(W)$  lysin to the P100 column under similar conditions as the protein standards. Effluent was analysed for lysin activity and the molecular weight of the enzyme determined using the  $V_e/\log$  molecular weight calibration curve.

#### PURIFICATION AND CONCENTRATION OF PHAGE LYSIN USING ULTRAFILTRATION

Ultrafiltration is a process in which membranes of a selected pore size are used to separate solute molecules on the basis of their molecular dimensions. The use of membrane filtration methods in enzyme purification has been reviewed (Porter, 1972). In this study ultrafiltration was employed to purify and concentrate the  $\phi C2(W)$ -induced lysin and to desalt enzyme preparations.

Ultrafiltration studies were undertaken using an Amicon Model 202 ultrafiltration cell (Amicon Corporation, Lexington, Mass. 02173, U.S.A.)

fitted with a range of ultrafiltration membranes. The capacity of the cell was about 200 ml but this was increased to about 1 l in some experiments by coupling a reservoir (Amicon product number RA500) to the cell. The unit was pressurised by nitrogen and was operated at 30 p.s.i. Unless stated otherwise ultrafiltration was undertaken at 5°C.

Ultrafiltration membranes were obtained from Amicon; Nuclepore (Nuclepore Corporation, Pleasanton, California 94566, U.S.A.); and Millipore (Millipore U.K. Ltd., Millipore House, London, England).

The chemical composition of the Amicon and Millipore membranes referred to in the text was given by Mr. Chainey and Mr. McGinty from Amicon and Millipore respectively.

All membranes were used in accordance with manufacturer's instructions. In most experiments the enzymes were desalted using 0.001 M potassium phosphate buffer pH 6.7 to wash out salts. In studies concerned with the effect of cations on lysin activation, enzyme preparations were desalted using distilled water.

#### EFFECT OF CATIONS ON THE LYTIC ACTIVITY OF $\phi$ C2(W)-INDUCED LYSIN

##### Monovalent cations

Stock solutions of sodium or potassium chloride were added to cells suspended in 0.01 M potassium phosphate buffer, pH 6.7, to give various  $\text{Na}^+$  and  $\text{K}^+$  concentrations. Cell suspensions were used to assay partially purified preparations of  $\phi$ C2(W) lysin and the effect of concentration of monovalent cations on the activity of the phage enzyme determined.

### Divalent cations

Stock solutions of calcium chloride, manganese sulphate, magnesium sulphate or cobalt chloride were added to cells suspended in 0.01 M  $\beta$  disodium glycerophosphate buffer, pH 6.7 to give a range of cation concentrations. Cell suspensions were used to assay partially purified phage lysin to enable the effect of divalent cations on the activity of the phage enzyme to be determined.

### EFFECT OF INHIBITORS ON THE LYTIC ACTIVITY OF $\phi$ C2(W)-INDUCED LYSIN

#### p Chloromercurobenzoate (CMBA)

The effect of CMBA on the lytic activity of  $\phi$ C2(W) lysin was studied by adding 200  $\mu$ l quantities of different concentrations of the inhibitor dissolved in 0.1 M phosphate buffer (pH 6.7) to tubes containing 200  $\mu$ l of partially purified  $\phi$ C2(W) lysin. After 5 min, 4 ml of a suspension of C10 cells in 0.1 M potassium phosphate were added to give an O.D.<sub>450</sub> of about 0.68 and the rate of cell lysis determined at 37°C.

#### Oxidised glutathionine

The effect of oxidised glutathionine on the lytic activity of the phage enzyme was determined as described for CMBA.

### SENSITIVITY OF SELECTED BACTERIA TO LYSIS BY $\phi$ C2(W)-INDUCED LYSIN

Selected strains were grown to about mid to late log phase and harvested by centrifugation. Cells were washed once in distilled water and resuspended in 0.1 M potassium phosphate, pH 6.7 and used to assay a partially purified phage lysin preparation essentially as described previously (p24) except that preparations of high lytic activity were used.

### Growth conditions

Streptococci (with the exception of *Str. thermophilus*), *E. coli* and *M. lysodeikticus* were grown in PLGYG broth. Group N streptococci and *M. lysodeikticus* were grown at 30°C. Other streptococci were incubated at 37°C. Lactobacillus, *Str. thermophilus* and leuconstoc strains were grown in MRS broth (De Man, Rogosa and Sharpe, 1960). Lactobacilli and the leuconstocs were incubated at 37°C and 30°C respectively, strains of *Str. thermophilus* were grown at 37°C.

### ACTION OF $\phi$ C2(W)-INDUCED LYSIN ON CELL WALLS

#### Preparation of cell walls

C10 cells were harvested from 1 x quantities of PLGYG broth (1% inoculum, 30°C, 6 h) by centrifugation and washed twice in distilled water. Washed cells were resuspended in distilled water to give thick suspensions. Cells were broken by sonicating 15 ml quantities of slurry using a Rapidis Ultrasonic Disintegrator fitted with a 9 mm tip. The instrument was operated at maximum power for 20 min and suspensions were cooled during disruption by immersion in a circulating ethylene glycol bath at 3°C. The disrupted cells were suspended in distilled water and centrifuged at 3,000 x g for 15 min to pellet unbroken cells and large particles. The supernatant was then centrifuged at 9,000 x g for 20 min at 4°C. The sediment (crude cell walls) was washed with 100 ml of 1 M sodium chloride and centrifuged at 17,000 x g for 20 min. The pellet was suspended in a saturated solution of sodium dodecylsulphate (SDS) and held at 37°C for 24 h. The cell walls were then sedimented by centrifugation at 17,000 x g for 20 min. The pellet was washed five times with distilled water and resuspended in distilled water. The cell walls were then centrifuged at 3,000 x g for 20 min and the supernatant

carefully removed. The cell walls were shell frozen at  $-30^{\circ}\text{C}$  and freeze dried. This procedure is a modification of the SDS method described by Wallinder and Neujahr (1971).

#### Identification of cell wall linkage attacked

Cell walls of C10 which had been purified by SDS treatment were suspended in 0.1 M potassium phosphate buffer pH 6.7 to give concentrations ranging from 1-1.5 mg/ml. Cell wall suspensions (5 ml) were incubated at  $37^{\circ}\text{C}$  in duplicate and partially purified  $\phi\text{C2(W)}$  lysin (100  $\mu\text{l}$ ) was added to one tube. Samples were removed from both tubes at intervals and analysed for reducing sugars, the liberation of free amino groups, and O.D.<sub>450</sub>. Reducing sugars were measured using the arsenomolybdate method described by Dische (1962). In this method  $\text{Cu}^{++}$  salts are reduced by sugars to  $\text{Cu}^{+}$  which reduces the arsenomolybdate to molybdane blue. The adsorption of the latter is directly related to the concentration of the sugar. Reducing sugars were calculated as glucose using the relationship derived by Dische (1962) that 50  $\mu\text{g}$  of glucose gives an optical density of 0.675 at 660 nm; 0.5 ml samples were used in analyses. Amino groups were determined using the fluordinitrobenzene (FDNB) method described by Ghuysen, Tipper and Strominger (1966). In this method FDNB is used to convert amino acids liberated by the action of lytic enzymes on the peptidoglycan to N-dinitrophenyl (DNP) derivatives. Amino groups liberated were expressed relative to alanine using the data of the previous workers who reported that the DNP derivative of alanine had a molar extinction coefficient of 5,200. The sample volume used was 0.3 ml.

### Chromatography of reduced enzyme digests of cell walls

Cell wall digests were reduced with borohydride, hydrolysed with hydrochloric acid and analysed for cell wall components using an amino acid analyser in an attempt to characterise  $\phi$ C2(W) lysin as an N-acetylglucosaminidase or an N-acetylmuramidase. Two millilitres of duplicate suspensions of C10 cell walls in 0.1 M potassium phosphate buffer, pH 6.7, were incubated in duplicate at 37°C. A range of cell wall concentrations (1 mg - 7 mg/ml) was used. To one tube 100  $\mu$ l of partially purified  $\phi$ C2(W) lysin were added. Tubes were held at 37°C for 6 h. At this time about 10  $\mu$ l of chloroform were added to each tube (to inhibit microbial growth) and held at 30°C for a further 14 h. Sufficient sodium hydroxide (1 M) was added to bring the pH to about 10.5-11 and 100  $\mu$ l of 10.5% (w/v) potassium borohydride were added to each tube. Tubes were then cooled to 5°C and held at that temperature for 24 h.

Three different procedures were then used to produce cell wall hydrolysates for analysis. In the first two methods the reduced samples were freeze-dried and the dried preparations acidified with 6 M hydrochloric acid, evacuated, sealed and hydrolysed at 110°C for 22 h in method A and for 4 h in method B, at 110°C. The hydrolysis conditions used in method A were similar to those used by Tsugita *et al.* (1968) who studied the action of T4 lysozyme on *E. coli* cell walls. In the third method the reduced cell wall preparations were not freeze-dried prior to hydrolysis and sufficient concentrated hydrochloric acid was added to the suspensions to give a 2.4 M solution of acid. The tubes were then evacuated, sealed and hydrolysed for 4 h at 95°C.

The hydrolysed material obtained from the three methods was freeze-dried to remove the acid and to concentrate the hydrolysed wall materials.



The dried hydrolysate was dissolved in 0.2 M sodium citrate buffer, pH 3.25. Samples for analysis were applied to the 65 cm x 0.9 cm column of the JLC 5AH amino acid analyser. The instrument was operated under similar conditions to those used for amino acid analysis (Spackman, Stein and Moore, 1958) except that a temperature of 45°C was used and the buffer was changed from pH 3.25 to pH 4.25 after 110 min. These conditions are similar to the amino acid analyser operating conditions used by Tsugita *et al.* (1968) in their study of the T4 lysozyme action on *E. coli*.

The amino acid analyser was calibrated using standard solutions of amino acids and amino sugars. The amino acid standard solutions were prepared by the technician who operated the analyser. Because the reduced products of muramic acid and glucosamine, muramicitol and glucosaminitol respectively were not commercially available, they were produced by reducing the appropriate amino sugar with borohydride essentially as described previously for the cell wall material. After reduction samples were hydrolysed with 6 M hydrochloric acid for 22 h at 110°C and freeze-dried. The freeze-dried material was then dissolved in sodium citrate. It was assumed that 100% conversion of amino sugar to alcohol occurred.

The concentration of amino sugar or other compound was calculated using the linear relationship between peak area and concentration. Peak area was calculated by multiplying the net height of a peak by the width of the peak at half height.

#### ULTRASTRUCTURAL STUDIES OF THE ACTION OF $\phi$ C2(W)-INDUCED LYSIN ON CELLS OF *Str. lactis* C10

Partially purified  $\phi$ C2(W) lysin was added to log phase cells of C10 suspended in 0.1 M potassium phosphate buffer pH 6.7 to give a lysin concentration of 300  $\mu$ /ml. Preparations were incubated at 37°C and samples

were removed at intervals. The lytic reaction was stopped by the addition of an equal volume of <sup>potassium</sup>phosphate buffered saline (PBS) containing 5% (v/v) glutaraldehyde. Fixed cells were pelleted by centrifugation at 3,000 x g, washed three times in PBS, and then post-fixed in 1% (w/v) osmium tetroxide for 1 h. The cells were again washed three times in PBS and dehydrated using a graded series of ethanols to 100%.

Gradients of hydroxy propyl methacrylate and finally Epon 812 resin were substituted for the ethanol and the resin was polymerised at 65°C for 72 h. Sections of thickness 60 nm were cut using an LKB Ultratome II, stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskopf 101 electron microscope using an accelerating voltage of 80 kV. The concentration of uranyl acetate used was 5% (w/v). The lead citrate was prepared by adding 1.33 g of lead nitrate and 1.76 g of sodium citrate to 30 ml of distilled water.

#### REAGENTS

With the exception of the materials used in electron microscopy and those noted in the text all reagents used were of 'Analar' or highest purity available and were obtained from BDH Chemicals, Poole, Dorset, England or from Sigma Chemicals, Fancy Road, Poole, Dorset. Reagents used in electronmicroscopy were supplied by Mr. John Parry, The Institute of Virology, Church Street, Glasgow and were obtained originally from Agar Aids for Electron Microscopy, Stanstead Road, Essex, England.

## RESULTS

### DEMONSTRATION OF BASIC NATURE OF LYTIC AGENT AND ITS HOMOGENITY

Preliminary experiments (Table 2.1) using a similar procedure to that reported by Oram and Reiter (1965) revealed that Amberlite CG50 (a weak carboxylic acid ion exchange resin) could be used to isolate and partially purify  $\phi$ C2(W) lysin. Lysin activity was eluted in one peak. Recovery of lytic activity, approaching 100% recovery, could have been obtained if all the lysin-containing fractions had been pooled. Some of the early fractions which were coloured and later fractions which had low enzyme activity were not pooled. These results show that the lytic agent in  $\phi$ C2(W) lysates behaves as a basic protein in chromatography on CG50 and suggests that the lytic activity present in  $\phi$ C2(W) lysates is caused by one agent.

Gel filtration on a column of Bio Gel P150 (exclusion limit 150,000) was used in an attempt to fractionate the lytic activity present in the CG50 effluent. Lytic activity was eluted as a single peak (Figure 2.1). High recovery of lytic activity was also found (Table 2.2). These results provide further evidence that the lytic activity in  $\phi$ C2(W) lysates is due to a single agent. Appreciable purification and removal of  $\phi$ C2(W) were also found suggesting that the combination of ion exchange chromatography and gel filtration could form the basis of a good purification scheme.

The void volume of the column, indicated by  $V_o$ , was determined using blue dextran 2000. The phage levels also peaked in this fraction. An estimate of the molecular weight of the lysin was obtained using the formula:-

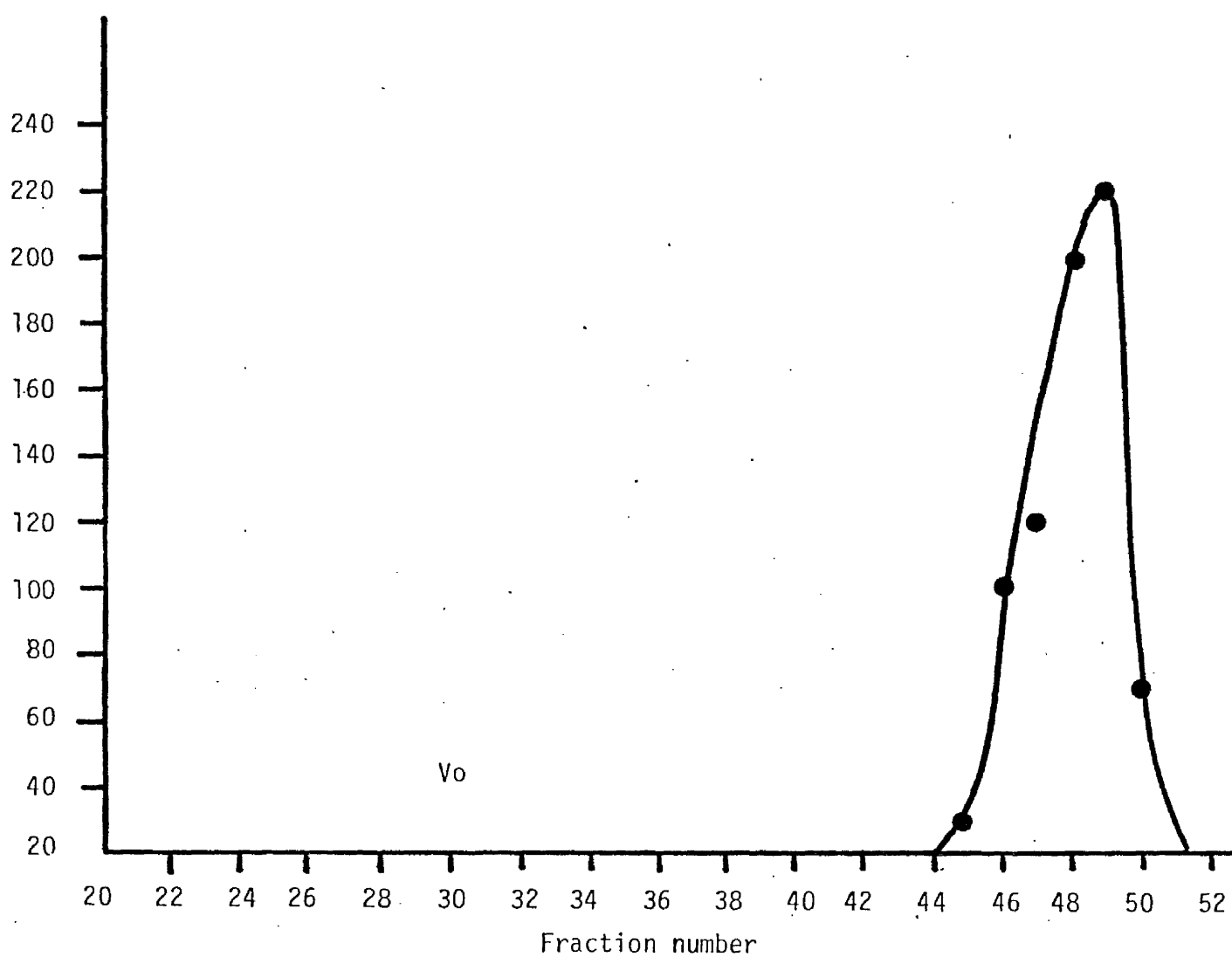
$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

Table 2.1

Isolation of lytic agent present in  $\phi$ C2(W) lysates by ion-exchange chromatography on Amberlite CG50

Material	Volume (ml)	Protein ( $\mu$ g/ml)	Phage (pfu/ml)	Lytic activity (u/ml)	Total lytic activity (u)	Specific activity	Purification	Yield (%)
Acetone treated lysate	3,900	500	$100 \times 10^8$	75	292,500	.15	1	100
Active fractions	136	380	$6 \times 10^8$	1,400	190,400	3.7	25	65.1

Figure 2.1      Chromatography of a diluted  $\phi$ C2(W) lysin preparation  
on a 89 cm x 2.6 cm column of Bio-Gel P100



—●—, lytic activity

Column operating conditions

Sample volume, 4.5 ml

Temperature, 5°C

Eluant, 0.1 M potassium phosphate, pH 6.7

Flow rate, 10.2 ml/h<sup>-1</sup>

Fraction volume, 5.1 ml

Table 2.2

Purification of  $\phi$ C2 lysin by gel filtration on Bio-gel P150<sup>(1)</sup>

Material	Volume (ml)	Lytic activity (u/ml)	Protein ( $\mu$ g/ml)	Phage (pfu/ml)	Total enzyme (u)	Total phage (pfu)	Yield (%)
Diluted effluent from CG50	4.5	400	260	$100 \times 10^7$	1800	$18 \times 10^{12}$	-
Active fractions from P150 column	37.5	30.5	$<10^{(2)}$	$28 \times 10^4$	2135	$10.5 \times 10^6$	118.6

<sup>(1)</sup>For conditions refer to Figure 2.2.<sup>(2)</sup>Protein level was too low to be determined accurately.

Where  $V_e$  = elution volume of lysin

$V_o$  = void volume

$V_t$  = total bed volume

to give a  $K_{av}$  value of 0.30 for the phage enzyme. This value was then used to obtain an approximate value of the molecular weight of  $\phi C2(W)$  lysin from the  $K_{av}$  plot against molecular weight given in the Bio Rad gel filtration laboratory manual (Anon., 1975) of about 50,000.

## ISOLATION AND PARTIAL PURIFICATION OF $\phi C2(W)$ -INDUCED LYSIN

### Preliminary experiments concerning the isolation of $\phi C2(W)$ lysin by ion exchange chromatography

Oram and Reiter (1956) used acetone precipitation as a preliminary step in the purification of  $\phi ML3$  lysin by ion exchange chromatography. This step was employed solely to reduce the phage level in the crude phage lysate. This procedure also reduced the phage level in  $\phi C2(W)$  lysates without significantly affecting the lysin concentration (Table 1.5). Acetone precipitation was accompanied by the appearance of a white flocculent precipitate essentially as described by the former workers. Since the phage level was significantly reduced and no significant reduction in protein levels was found it was initially assumed that the precipitate was phage. Because of safety considerations (acetone is inflammable and forms a potentially explosive mixture with air) and the difficulties of using acetone precipitation with large volumes of broth lysates, experiments were undertaken to isolate  $\phi C2(W)$ -induced lysin without using acetone precipitation. Studies were initiated using 30 x 2.5 cm columns of CG50 or Bio Rex 70 (20-50 mesh). The columns were equilibrated with 0.1 M potassium phosphate buffer pH 5.8 and elution was undertaken with 0.2 M K-phosphate buffer pH 7.3. It was found that the columns had a relatively low capacity for lysin. Lysin binding sites were saturated by about 1  $\mu$ l of lysate. Consequently large quantities of ion exchange resin would be required

to isolate litre quantities of partially purified lysin. This was unexpected and the possibility that acetone precipitation removed medium constituents which would otherwise compete with lysin for binding sites on the resins was considered. In a further experiment in which 9  $\mu$ l of a diluted PLGYG broth lysate (50% v/v dilution with distilled water and adjusted to pH 5.8) were applied in the descending mode to a 30 x 2.5 cm column of Bio Rex 70 (20-50 mesh) and eluted in the ascending mode (a procedure which concentrates materials absorbed on the resin) a white crystalline precipitate was present in the enzyme containing fractions. The fractions containing the highest level of precipitate (estimated visually) occurred past the fractions containing the highest concentration of lysin. This observation suggests that the precipitated material(s) is/are more basic than the lysin.

If a practical method could be found to increase the lysin binding capacity of the resins then the requirements for ion exchange resins would be reduced and laboratory size columns could be used in enzyme purification. Initially it was thought that the precipitated material was a basic medium constituent and attempts were made to find a medium which contained a lower level of substances competing for resin binding sites. It was envisaged that if this medium was used to prepare phage lysates, the ion exchange resins should exhibit an increased capacity to bind the phage-induced enzyme. The effect of producing  $\phi$ C2(W) lysates in different media on the apparent capacity of Bio Rex 70 for lysin is shown in Table 2.3.

The tryptone yeast extract medium was found to give highest lysin binding. This finding was confirmed in repeat experiments. That the capacity of Bio Rex 70 for phage lysin was not simply related to medium composition is also apparent. Similar lysin binding occurred in PLGYG



Table 2.3

Effect of medium on the apparent capacity of Bio Rex 70  
for  $\phi$ C2(W) lysin

Medium	Lysin bound (u)
TYGG	3,200
TLBC	900
PLGYG	1,500
50% m PLGYG	1,500

1 g of Bio Rex 70 (20-50 mesh) was equilibrated with 0.05 M K-phosphate buffer pH 5.8, mixed with 30 ml of the respective broth lysin preparation and the quantity of lysin bound was calculated.

and in a medium designated 50% PLGYG which contained half the concentration of medium components. This suggested that the material or material(s) competing with lysin could be produced, at least in part, by the phage-infected cells or perhaps by uninfected cells of C2.

This hypothesis was tested by adding 40% (v/v) acetone to uninoculated PLGYG and tryptone yeast extract media and to phage lysates prepared in both media. No precipitate was found in the uninoculated media but was present in the phage lysates. The cell free supernatant of control log phage cultures of C2 (not infected with  $\phi$ C2(W)) also gave a precipitate with 40% (v/v) acetone. This suggests that the material(s) which compete with lysin for absorption sites on the ion exchange resins is/are produced at least in part by C2.

The white crystalline material present in fractions from the ion exchange experiment discussed previously was removed from the eluant buffer by filtration and washed several times with 40% (w/v) acetone. The material was only slightly soluble in water or dilute or concentrated solutions of sodium hydroxide. It was very soluble in dilute or concentrated acids. A saturated solution of the material in distilled water at pH 6 when tested for antibacterial activity against *B. stearoothermiphilus* using the modified Disc Assay method as described by Crawford and Galloway (1964), gave a zone of inhibition of about 12 mm. The antimicrobial activity was not destroyed by boiling for 5 min. The material passed through an Amicon YM20 ultrafiltration membrane (exclusion limit 20,000) suggesting that it had a molecular weight of <20,000. A 0.1% w/v solution of the material in 0.1 M hydrochloric acid gave a negative reaction for protein with the Bio Rad protein assay method.

### Large scale isolation and partial purification of $\phi$ C2(W)-induced lysin

Following small scale isolation experiments an empirical procedure was developed which gave a good yield and purification of the lytic enzyme. This procedure was scaled up and used to isolate the enzyme present in 108  $\mu$ l of tryptone yeast extract broth lysate (Table 2.4). Elution yielded about 10  $\mu$ l of concentrated partially purified lysin. The 17-fold purification obtained was significantly lower than that achieved in the smaller scale experiment shown in Table 2.1. Significant phage removal was also obtained. The yield figure was of interest. Significantly higher recovery of lytic activity than that present in broth lysate was obtained (131%). This phenomenon, the apparent recovery of more lytic activity, than that present in the crude lysate was found occasionally in small scale purification experiments and may have been due to the removal of an inhibitor or the disaggregation of enzyme. The pooled fractions were stored at  $-30^{\circ}\text{C}$  until required for further purification. Enzyme levels were stable at  $-30^{\circ}\text{C}$  for at least 2½ years.

### Further purification of CG50 eluate by ultrafiltration

Experiments were performed using a range of ultrafiltration membranes of different chemical composition and exclusion limits (nominal molecular weight limit). Experiments were performed using an Amicon, Model 202 ultrafiltration cell (membrane area 27.5 cm<sup>2</sup>) fitted with different membranes in which 50 ml of lysin was concentrated to about 1-3 ml and the concentrate and ultrafiltrate tested for lysin. No lytic activity was recovered from concentrates produced using polysulphone containing membranes such as the Amicon PM series or the Millipore PT series membranes. Up to 50% of the lytic activity was lost if the polyoelifine containing membranes (Amicon XM50, XM100, XM300) were used. Little or no loss of lytic activity

Table 2.4

Large scale isolation, concentration and purification of  
 $\phi$ C2(W) lysin on CG50

Lysate

Volume (ℓ)	=	108
Enzyme (u/ml)	=	240
Total enzyme (u)	=	$25.9 \times 10^6$
Phage (pfu/ml)	=	$4 \times 10^{10}$
Protein (μg/ml)	=	480
Specific activity	=	.5
Total phage (pfu)	=	$43.2 \times 10^{14}$

Active fractions

Volume (ml)	=	9,990
Enzyme (u/ml)	=	$3.4 \times 10^3$
Total enzyme (u)	=	$33.95 \times 10^6$
Protein (μg/ml)	=	398
Phage (pfu/ml)	=	$2 \times 10^{10}$
Specific activity	=	8.54
Purification	=	17.1
Total phage (pfu)	=	$19.98 \times 10^{11}$
Yield (%)	=	131

occurred if polysaccharide type membranes such as Amicon YM10, YM30; Millipore PSAC, PSVP; Nucleopore type C series membranes or the non-ionic, synthetic, Amicon UM20 membrane were used.

Although it was believed that the loss of lytic activity in concentrates produced using the polysulphone and polyoelifine containing membranes was due to adsorption effects, attempts to prove this were unsuccessful. Lysin could not be eluted from PM10 membranes with 0.2 M potassium phosphate buffer, pH 7.3 or with 75% (v/v) ethylene glycol. The ability of polysulphone containing membranes to 'adsorb' lysin was not restricted to partially purified lysin preparations and lysin present in crude lysates was also absorbed. Ultrafiltration using polysulphone membranes could not be used to produce lysin free, high titre phage preparations because phage was bound also.

The use of ultrafiltration to concentrate and further purify the  $\phi$ C2(W) lysin present in the CG50 eluant is shown in Table 2.5. In this particular experiment a further 18-fold purification was obtained giving a final purification figure of about 200. It must be emphasised that the 60 ml concentrate had to be washed four times with an equal volume of 0.05 M potassium phosphate buffer, pH 6.7 each time to obtain this degree of purification. In other experiments in which smaller volumes of eluate (100-200 ml), were used higher purification figures, up to 30-fold, were sometimes obtained.

Although ultrafiltration with the extremely hydrophilic YM30 membrane resulted in significant enzyme purification and marked concentration of enzyme activity, the phage level was also proportionately increased. Attempts were made to reduce the phage level by using an ultrafiltration membrane of sufficiently large pore size to allow the lysin to pass

Table 2.5

Concentration and further purification of  $\phi$ C2(W) lysin  
by ultrafiltration

Partially purified CG50 material

Volume (ml)	500
Protein ( $\mu$ g/ml)	430
Phage (pfu/ml)	$2 \times 10^{10}$
Lytic activity (u/ml)	2,400
Total lytic activity (u)	$1.2 \times 10^6$
Specific activity	5.58

Washed concentrate<sup>(1)</sup>

Volume (ml)	60
Protein ( $\mu$ g/ml)	154
Phage (pfu/ml)	$18 \times 10^{10}$
Lytic activity (u/ml)	15,750
Total lytic activity (u)	$0.945 \times 10^6$
Specific activity	102.3
Purification	$18.3 (205)^{(2)}$
Yield (%)	79

(1) Lysin preparation was concentrated using YM30 ultrafiltration membrane. Concentrate was washed four times with an equal volume of quarter strength Ringer solution.

(2) Final purification achieved compared with starting lysate (see Table 2.4).

through but not the phage. The effect of using the Millipore PSVP membrane (exclusion limit  $10^6$ ) to reduce the phage level is shown in Table 2.6. Only a slight reduction in lysin concentration was found with a large reduction in phage concentration (99.5%). However, high concentrations ( $2 \times 10^8$  pfu/ml) still remained. The effect of using another membrane with an exclusion limit of 50,000 is also shown in Table 2.6. Although this membrane yielded an ultrafiltrate with a lower phage level it was necessary to wash the membrane with buffer to obtain even a modest recovery of enzyme. If a lower exclusion limit membrane (YM30) was used to concentrate the activity present in the ultrafiltrate to that present in the PSVP experiment, no advantage of phage reduction was attained since similar phage levels were present in the concentrate. These results reflect the findings of a large number of ultrafiltration experiments. It is established that ultrafiltration can be used to obtain a significant reduction in phage levels but not to obtain an enzyme preparation free of phage.

#### Partial purification by gel filtration

In preliminary experiments the lytic enzyme in CG50 effluent was concentrated and partially purified by ultrafiltration using an Amicon YM30 membrane and applied to a column of Bio Gel P100. This method yielded enzyme purified from 300-500 fold depending upon the sample volume applied to the column. In later experiments a more rapid method which facilitated the handling of larger quantities of lysin was developed.

It was established that the  $\phi$ C2(W)-induced lysin was precipitated by the addition of sufficient ammonium sulphate to give a solution of 30-50% saturation. Lysin was concentrated in good yield by the addition of ammonium sulphate to 46% saturation. This procedure increased the

Table 2.6

Use of ultrafiltration to reduce the phage level in  
preparations of  $\phi$ C2(W)-induced lysin

	Membrane type	
	PSVP	Nucleopore $10^5$ <sup>(1)</sup>
<u>Partially purified CG50 material</u>		
Volume (ml)	200	200
Phage (pfu/ml)	$2 \times 10^{10}$	$2 \times 10^{10}$
Lytic activity (u/ml)	2,340	2,340
<u>Ultrafiltrate</u>		
Volume (ml)	150	150
Phage (pfu/ml)	$2.2 \times 10^8$	$1.4 \times 10^7$
Lytic activity (u/ml)	3,250	320
% reduction in phage concentration	99.5	99.9

(1) Exclusion limit  $10^5$  daltons.



specific activity by about 2-fold. The phage level was also increased (Table 2.7). Application of the precipitate dissolved in elution buffer to a column of Sepharcryl S200 resulted in significant enzyme purification and a reduction in the phage titre. The final lysin purification figure depended upon the sample volume applied. Higher sample volumes resulted in lower purification figures. For lower volumes the situation was reversed. The pooled fractions from the gel filtration column were frozen after concentration and desalting by ultrafiltration using the YM30 membrane.

Examination of the gel filtration results (Figure 2.2) shows that the lysin peak was well separated from the void volume peak (phage  $\phi$ C2(W) peaks in this fraction). Although gel filtration resulted in a 99.988% reduction in the phage level in the active fractions (not allowing for dilution) the pooled fractions still contained high levels of  $\phi$ C2(W). It is apparent from Figure 2.2 that considerable UV-absorbing material is removed by gel filtration and that a UV peak corresponding with lysin activity was not obtained. The pooled fractions from the column therefore contained at least one impurity in addition to  $\phi$ C2(W); they contained material from peak B. Since enzyme purified by this procedure was extensively used throughout this study electron microscopy was used in an attempt to determine whether the lysin was associated with phage components. No evidence of phage-associated structures i.e. tails or heads was found in lysin preparation produced by the above method.

Lysin produced by this method was relatively unstable. The stability apparently depended upon the protein concentration; the lower the protein concentration the more unstable the preparation. With some preparations, about 50% activity was lost on storage at 5°C for 24 h. Lytic activity was stable, however, at -30°C. Aged preparations could not be reactivated by 10 mM concentrations of cysteine or mercaptoethanol. These compounds

Table 2.7

Further purification of  $\phi$ C2(W) lysin by ammonium sulphate precipitation and gel filtration

Material	Volume (ml)	Protein ( $\mu$ g/ml)	Phage (pfu/ml)	Lysin (u/ml)	Total lysin (u)	Specific activity	Purification <sup>(1)</sup>	Lysin yield (%)(2)
CG50 active fractions	440	360	$4 \times 10^{10}$	$3.336 \times 10^3$	$1.001 \times 10^6$	9.27	18.5	100
Ammonium sulphate precipitate	27	1540	$12.5 \times 10^{11}$	$27.552 \times 10^3$	$0.342 \times 10^6$	17.8	35	34
Active fractions from S200 column	90	25	$14.7 \times 10^7$	$3.336 \times 10^3$	$0.250 \times 10^6$	133.4	267	25

131

<sup>(1)</sup> Compared with broth lysate - see Table 2.4.<sup>(2)</sup> Based on lytic activity present in CG50 active fractions.

Figure 2.2      Chromatography of a partially purified (ion-exchange and ammonium sulphate precipitational  $\phi$ C2(W) lysin preparation on a 90 cm x 2.6 cm column of Sephacryl S200

Column operating conditions

Sample volume, 25 ml

Eluant contained 0.1 M K-phosphate, 5 mM

E.D.T.A., 5 mM mercaptoethanol and 0.01% (w/v)  $\text{NaN}_3$

Flow rate, 44 ml  $\text{h}^{-1}$

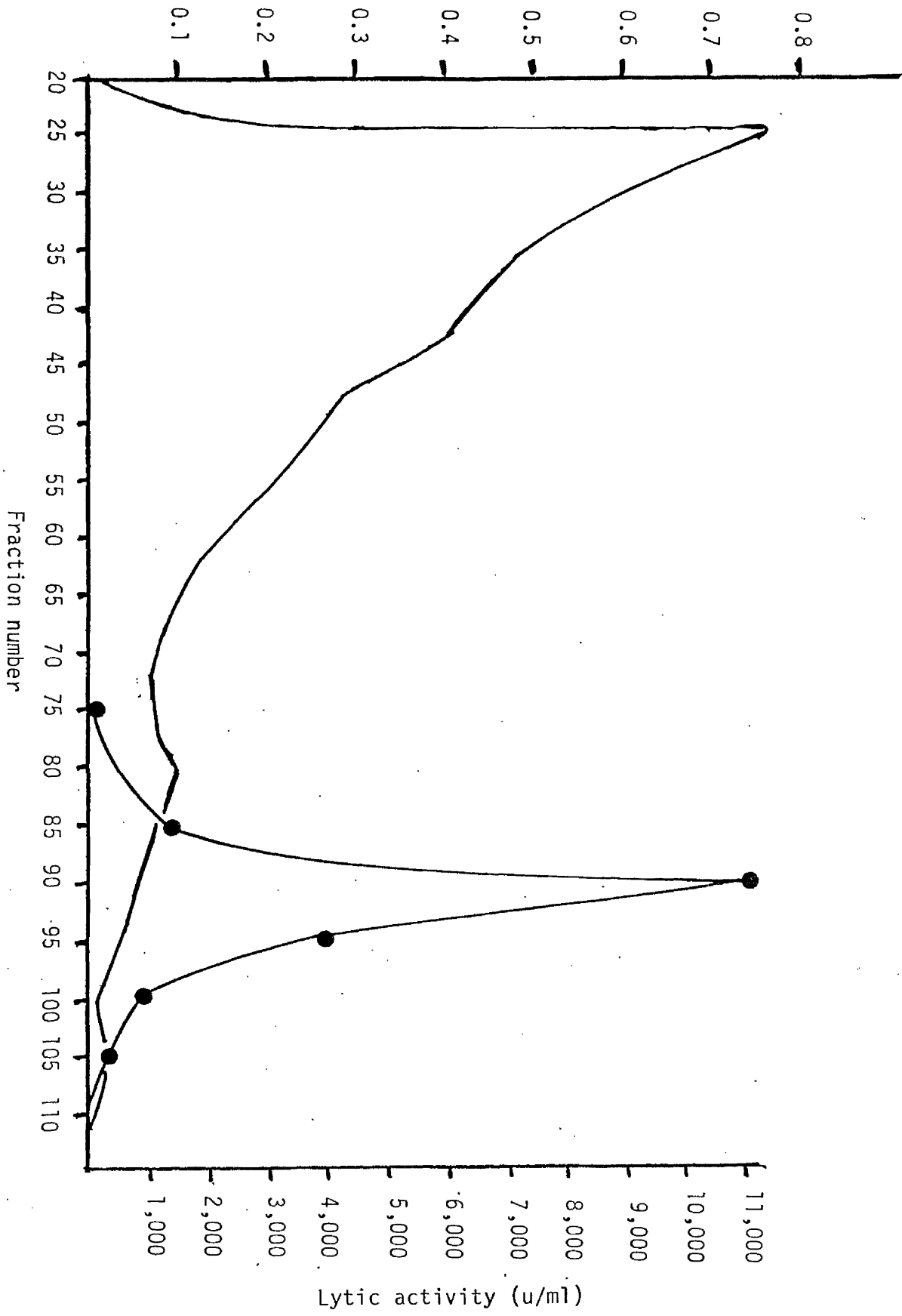
Fraction volume, 5.86 ml

Temperature, 5°C

—, absorbance 280 nm

—●—, lytic activity

Figure 2.2



did not stimulate lytic activity either. Attempts to further purify preparations using ion-exchange chromatography were unsuccessful and resulted in loss of activity.

#### Dye ligand chromatography

Attempts to isolate and purify phage lysin by dye ligand chromatography using the Amicon Dye Matrix protein purification kit were unsuccessful. All four ligands in addition to the unsubstituted agarose control bound the lysin (Table 2.8) but only low enzyme recoveries were obtained using a variety of elution techniques including 0.1 M SCN or 75% w/v ethylene glycol. It was of great interest to find that the green A ligand bound the lysin but not the phage. The potential importance of this find is apparent. If an elution method could be developed to elute lysin from the green A ligand then a method for the production of phage-free lysin would be available.

#### Affinity chromatography

Attempts to purify the enzyme by affinity chromatography on deaminated chitin (DECH) by the method of Weaver *et al.* (1977) were also unsuccessful. Since this method was developed for the purification of hen egg lysozyme which is markedly different than  $\phi$ C2(W)-induced lysin the results were perhaps not surprising.

#### PROPERTIES OF PARTIALLY PURIFIED $\phi$ C2(W)-INDUCED LYTIC ENZYME

Characterisation experiments were mainly undertaken with enzyme preparations which were purified by ion exchange chromatography, ammonium sulphate precipitation, and gel filtration. In some experiments the enzyme preparation used was purified by ion exchange chromatography and differential ultrafiltration i.e. the CG50 eluate was passed through a

Table 2.8

Attempted purification of  $\phi$ C2(W)-induced lysin by dye-ligand chromatography using the Amicon dyematrix screening kit

Column	Washings		Eluate	
	Phage (pfu/ml)	Lytic activity (u/ml)	Phage (pfu/ml)	Lytic activity (u/ml)
Control	$2 \times 10^8$	<1	$7.9 \times 10^9$	6
Blue A	$9.7 \times 10^9$	<1	$9.4 \times 10^9$	7
Red A	$1.3 \times 10^8$	<1	$1.28 \times 10^{10}$	16
Orange A	$8 \times 10^8$	<1	$1.30 \times 10^8$	4.4
Green A	$1.26 \times 10^{10}$	<1	$<1 \times 10^6$	7
Blue B	$2 \times 10^8$	<1	$1.66 \times 10^{10}$	5

Columns were equilibrated with 20 mM Tris/HCl buffer pH 7.5, 500  $\mu$ l of partially purified lysin were applied to each column. The preparation contained 2620 u of lytic activity and  $5.5 \times 10^{10}$  pfu of  $\phi$ C2(W). Columns were washed with 10 ml of equilibration buffer and eluted with 10 ml of equilibration buffer containing 1.5 M KCL.

Millipore PSVP filter to reduce the phage level and desalted and further purified by ultrafiltration using a YM30 membrane. No apparent differences were noted in the enzymes prepared by either method.

#### Effect of pH

The lytic enzyme was active over the pH Range 5-8 with an apparent pH optimum of 6.5-6.9 (Figure 2.3). No lytic activity was apparent at pH 4.5 or pH 9.0.

#### Effect of temperature

The effect of temperature on the activity of the lysin is shown in Figure 2.4. The lysin had an apparent temperature optimum of 37°C and had a Q10 of 2.5 in the range 22°-32°C. However, the Q10 for the range 32-42°C was only 0.8.

Activation energies have been reported for some streptococcal phage lysins (Oram and Reiter, 1965; Tourville and Tokuda, 1967). The activation energy of an enzyme can be calculated using an integrated form of the Arrhenius rate equation:-

$$\ln k = \ln A - \frac{E_a}{RT}$$

Where k = some measure of reaction velocity

A = constant

E<sub>a</sub> = activation energy

T = absolute temperature

R = gas constant.

A plot of  $\ln k$  against  $\frac{1}{T}$  should be a straight line, with a slope of  $-\frac{E_a}{R}$

Figure 2.3      Effect of pH on the activity of partially purified  $\phi$ C2(W) lysin

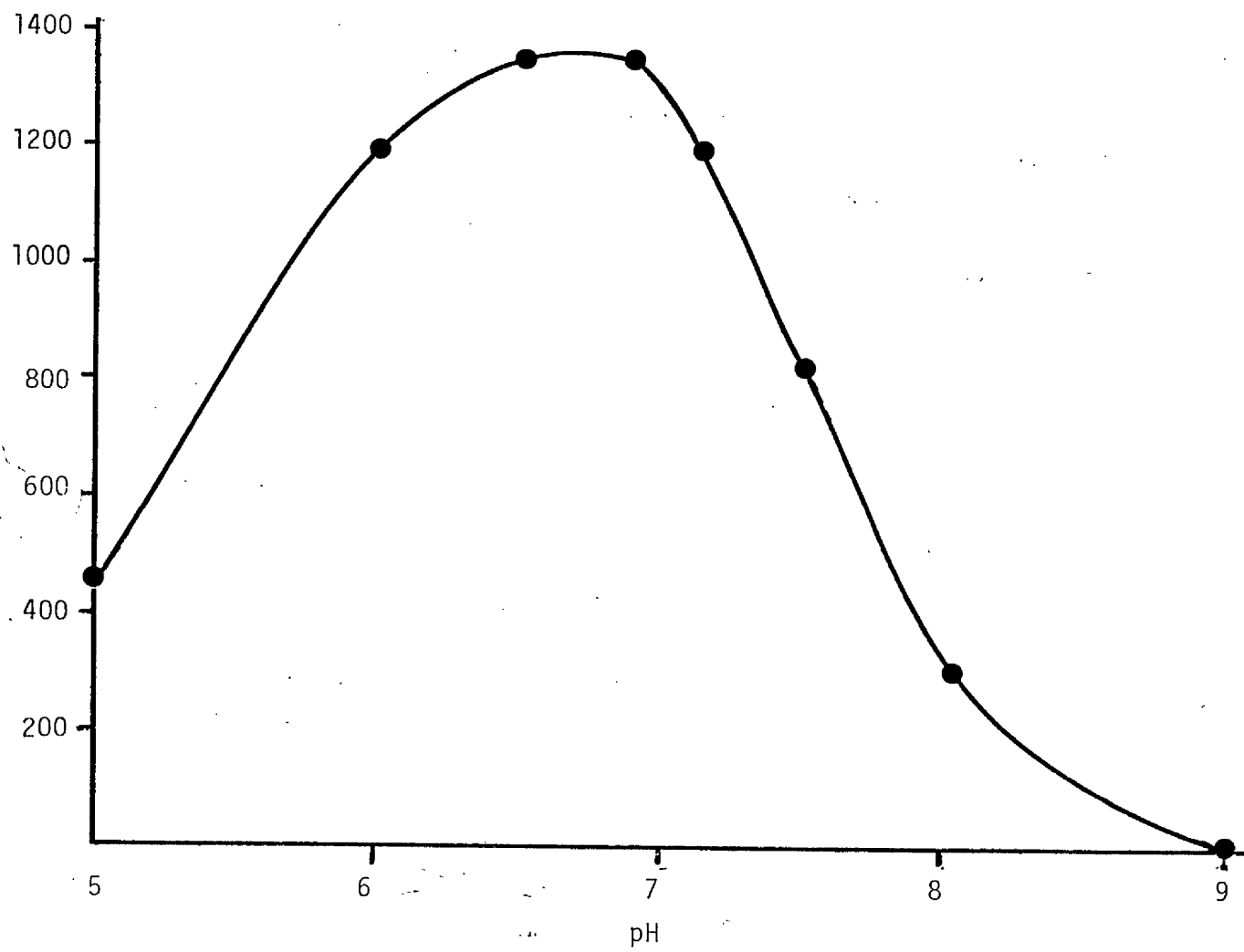
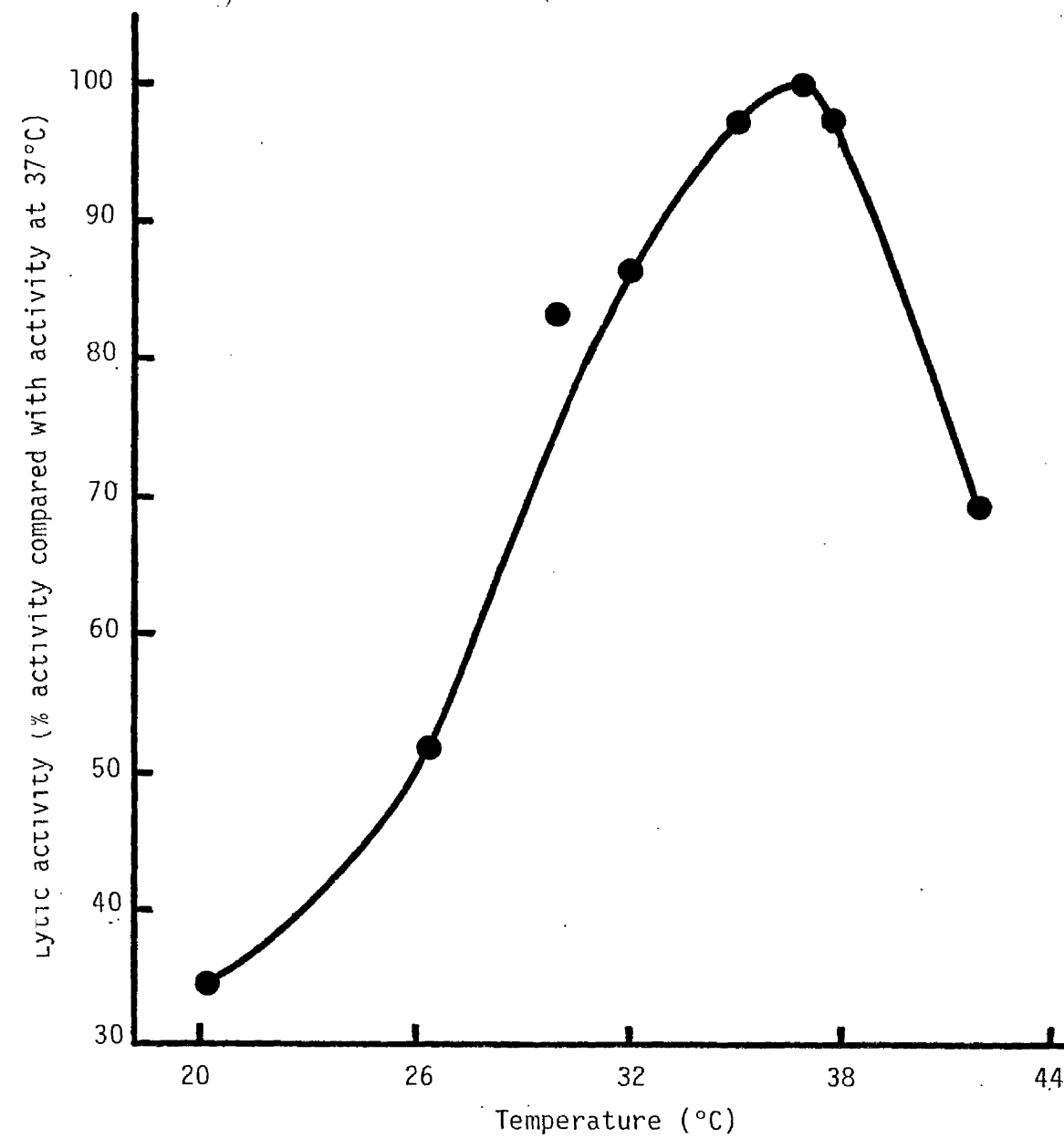




Figure 2.4 Effect of temperature on the activity of  $\phi$ C2 lysin



A plot of  $\ln k$  against  $\frac{1}{T}$  for the data present in Figure 2.4 is shown in Figure 2.5. Calculation of  $E_a$  revealed a value of  $16.5 \text{ kcal mole}^{-1}$  ( $69.2 \text{ kJ mole}^{-1}$ ) for the phage lysin over the temperature range 22-32°C.

The effect of temperature on the inactivation of  $\phi C2(W)$ -induced lysin is shown in Figure 2.6. From the results it is apparent that the lysin was unstable at temperatures above 30°C. After 5 min at 47°C only 3.4% of the activity present in the preparation held at 30°C remained. For comparison the effect of temperature on the inactivation of  $\phi C2(W)$  was also studied (Figure 2.7). Unlike the previous experiment,  $\beta$ -disodium glycerophosphate buffer was used because several workers (reviewed by Adams, 1959) have reported that phosphate may accelerate the thermal inactivation of phages. It is apparent that the virus was relatively insensitive to thermal inactivation compared with the phage lysin and showed little drop in titre after 5 min at 60°C, the phage lysin was inactivated by this treatment. Considerable inactivation of  $\phi C2(W)$  occurred at 65°C and complete inactivation was obtained after 5 min at 73°C.

#### Effect of cations

The phage enzyme was activated by both monovalent and divalent cations. The effect of  $\text{Na}^+$  and  $\text{K}^+$  on activity is shown in Figures 2.8 and 2.9. Maximum lytic activity was found with 0.1 M concentrations of either ion. Higher concentrations gave reduced activity. With  $\text{Na}^+$ , lytic activity fell to a constant value which was independent of cation concentration over the range 0.2-0.4 M. A similar effect was also observed with  $\text{K}^+$  over the concentration range 0.2-0.3 M. A sharp peak of lytic activity was found for  $\text{Mn}^{++}$ ,  $\text{Ca}^{++}$  and  $\text{Co}^{++}$  at ionic concentrations 3, 5 and 8 mM respectively (Figures 2.12, 2.10, 2.13). Complete inhibition of lytic activity was found with 30 mM  $\text{Co}^{++}$ . With  $\text{Mg}^{++}$  (Figure 2.11),

Figure 2.5 Arrhenius plot showing the effect of temperature on the activity of  $\phi\text{C}_2(\text{W})$  lysin

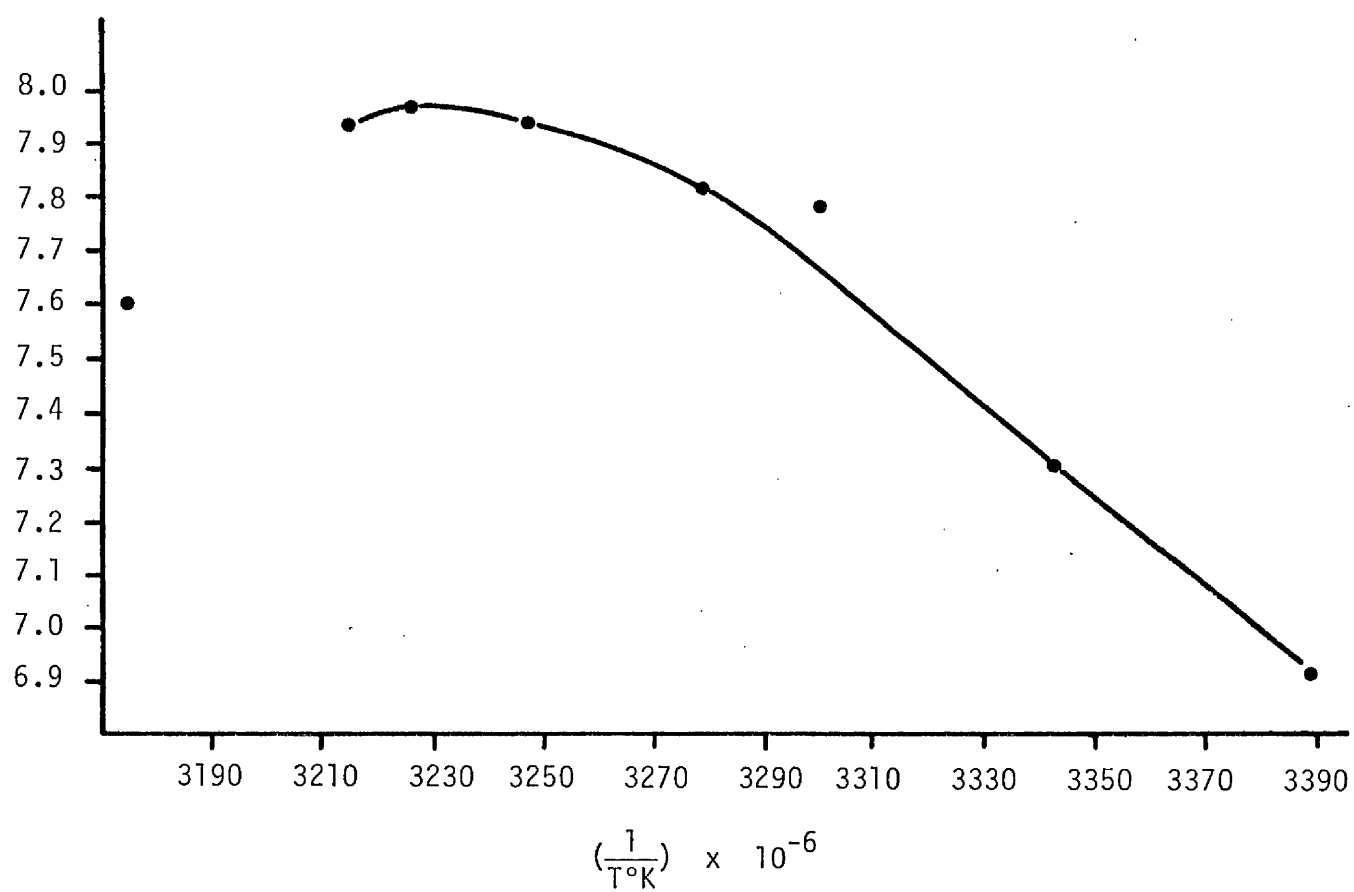


Figure 2.6      Stability of partially purified  $\phi$ C2(W) lysin at various temperatures.

The enzyme was incubated for 5 min in 0.1 M potassium phosphate buffer pH 6.7 at each temperature indicated. Activities are expressed as a percentage of the activity at 30°C

Figure 2.7      Stability of  $\phi$ C2(W) at various temperatures

The phage was incubated in 0.01 M  $\beta$ -disodium glycerophosphate buffer, pH 6.7 for 5 min at each temperature indicated. After this time tubes were removed, cooled rapidly to <10°C and titred for survivors.

Figure 2.6

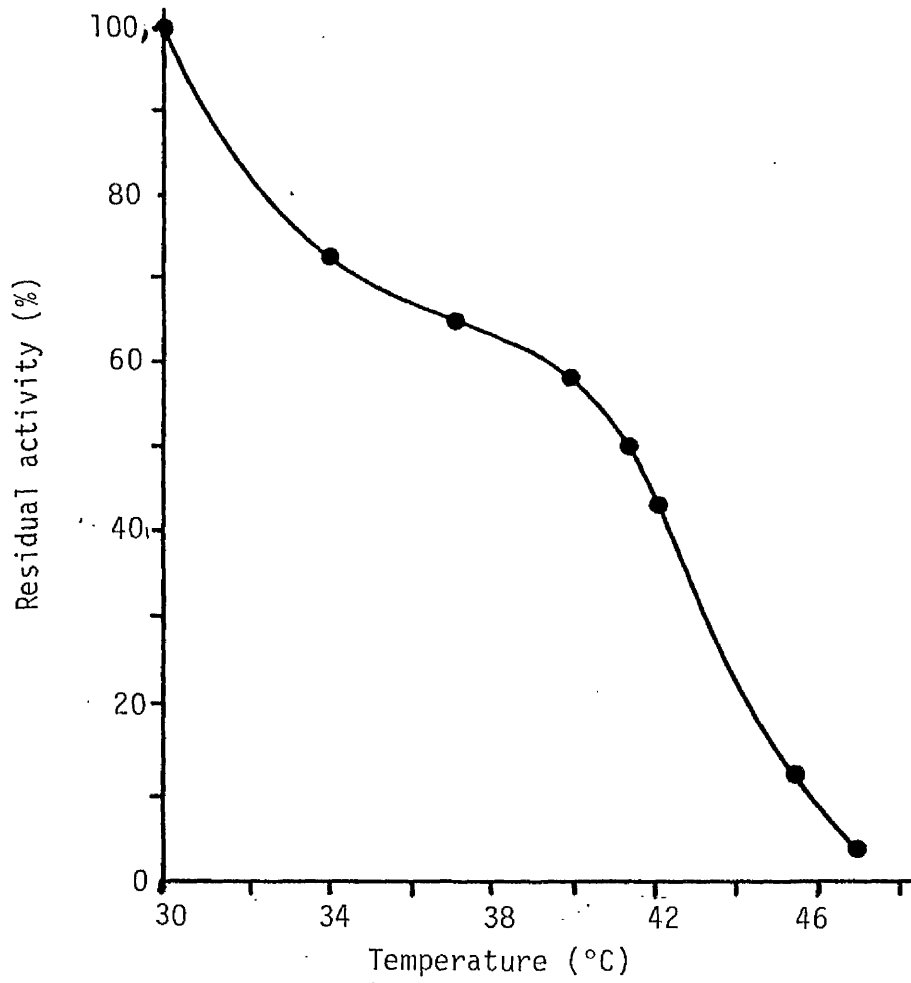


Figure 2.7

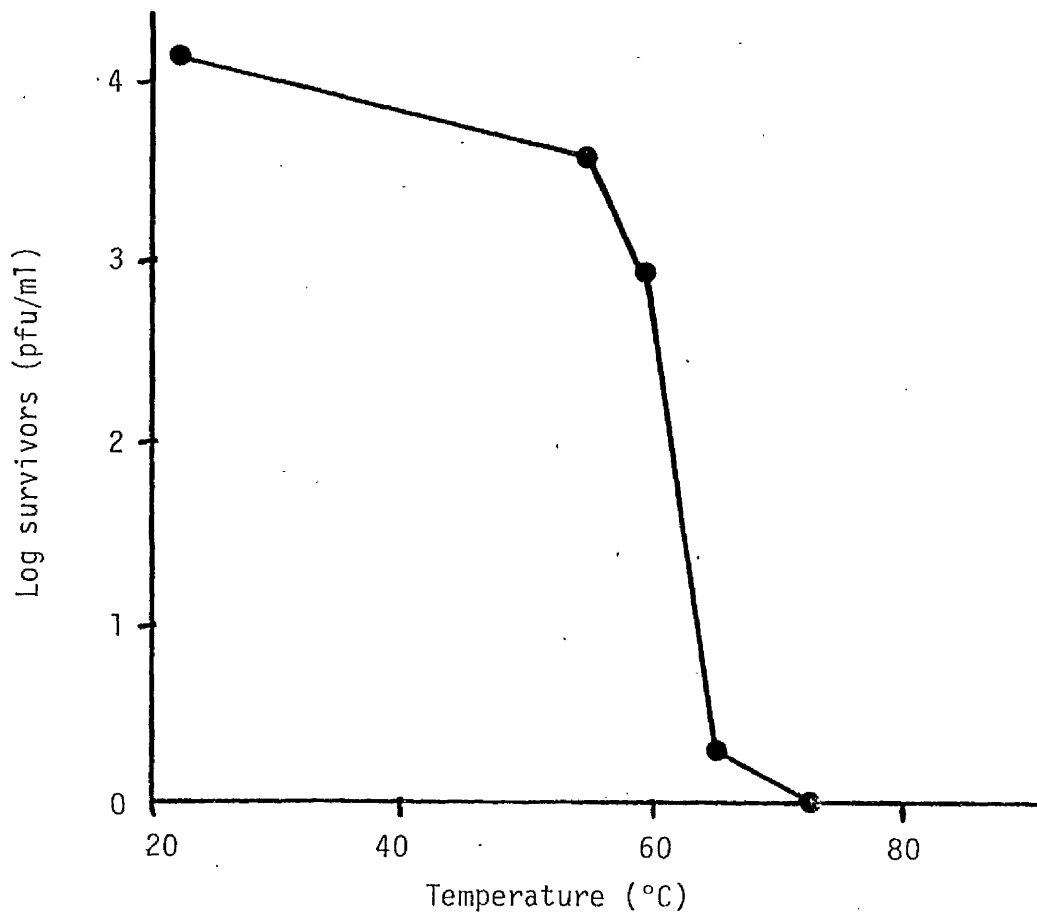


Figure 2.8 Effect of  $\text{Na}^+$  on the activity of  $\phi\text{C2(W)}$

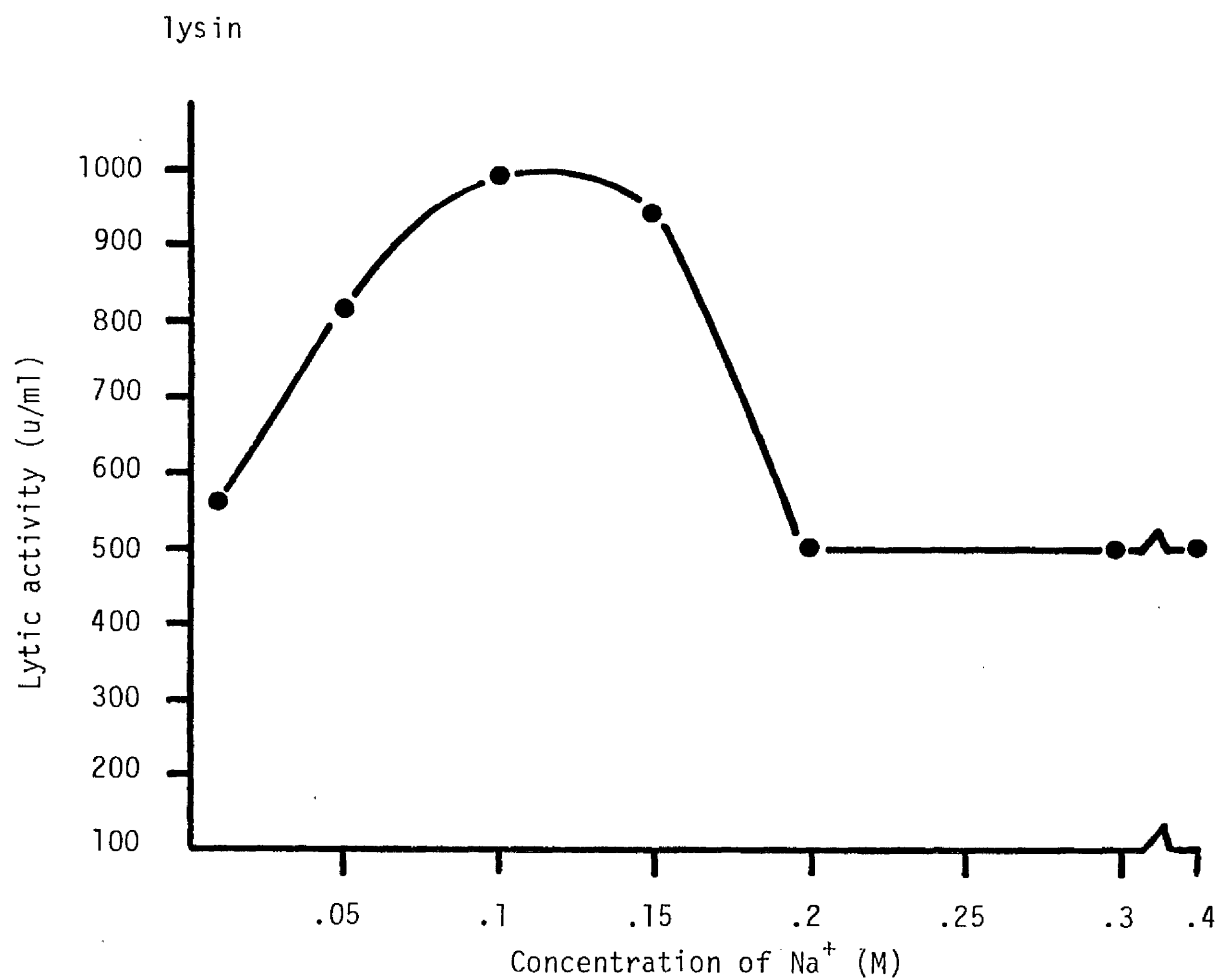
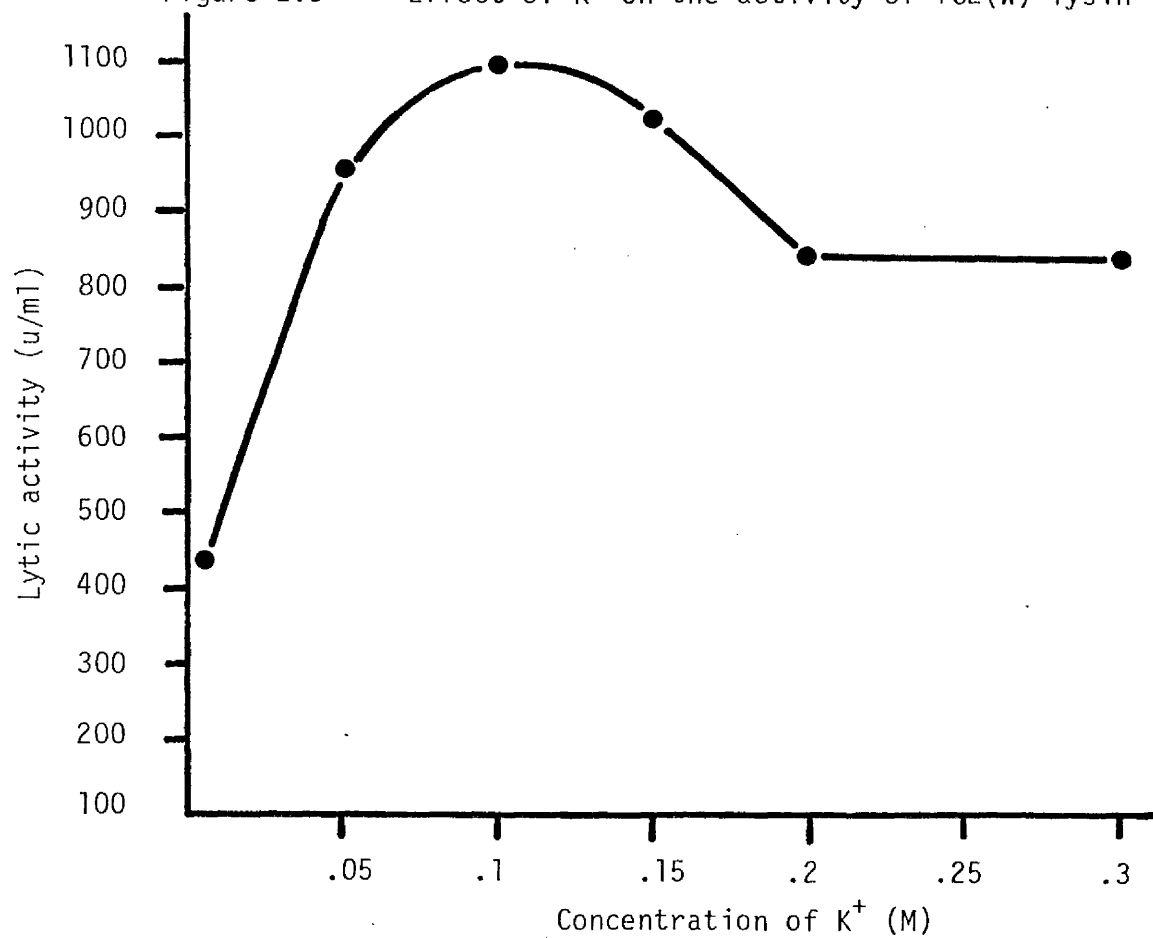


Figure 2.9 Effect of  $\text{K}^+$  on the activity of  $\phi\text{C2(W)}$  lysine



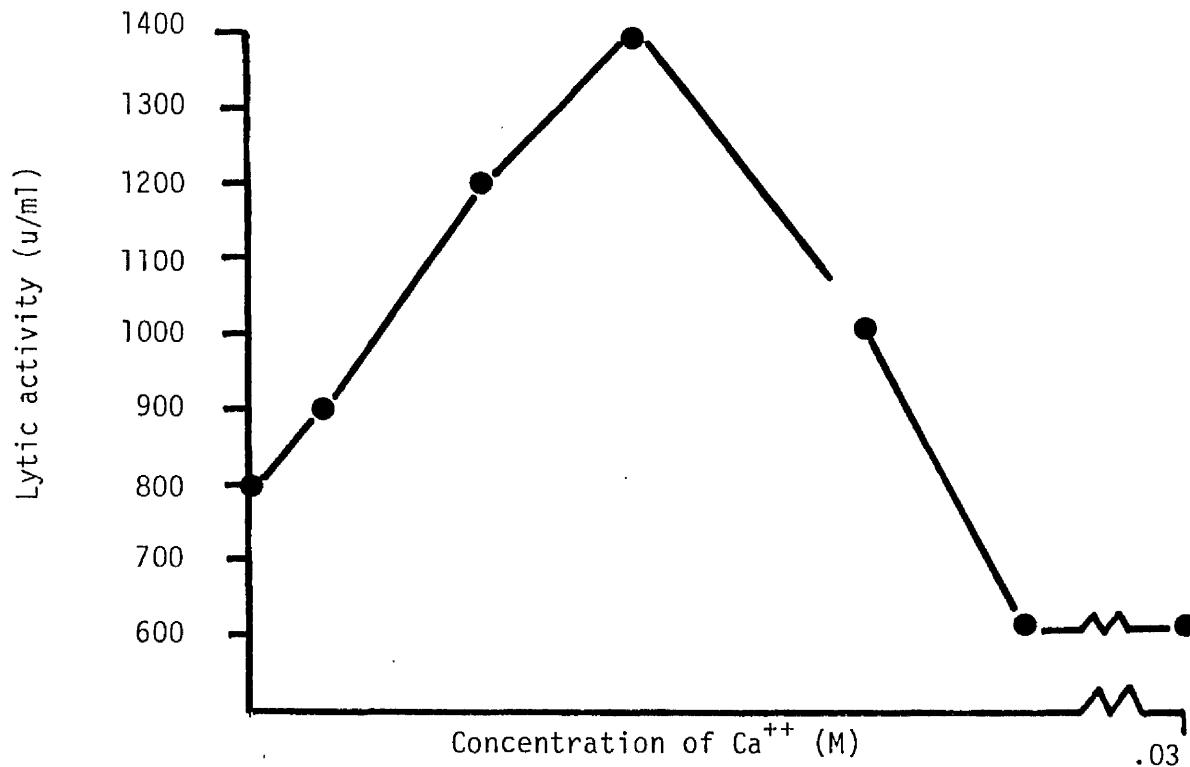


Figure 2.11 Effect of  $\text{Mg}^{++}$  on the activity of  $\phi\text{C2(W)}$  lysin

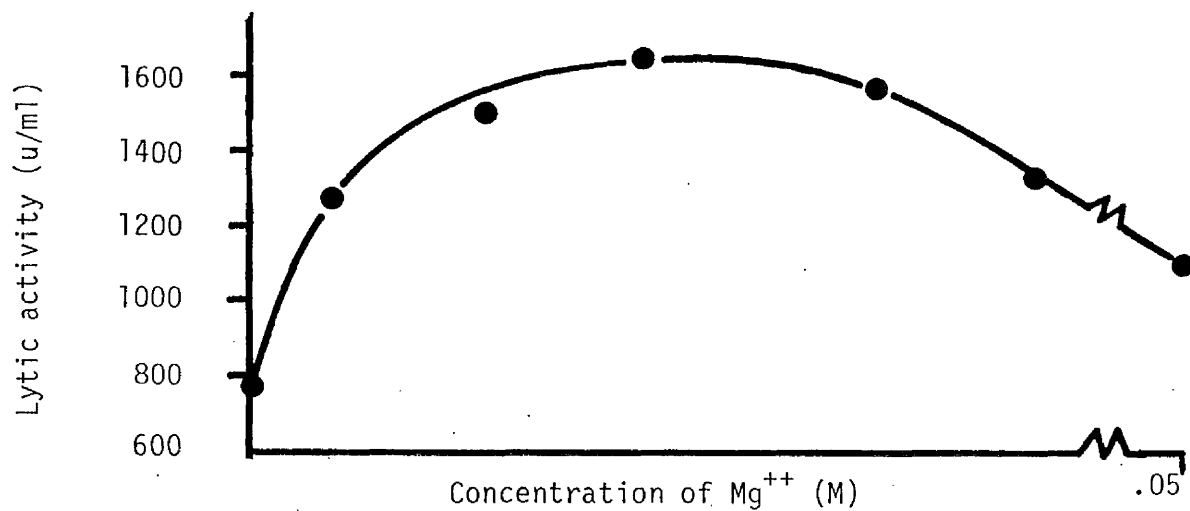


Figure 2.12 Effect of  $\text{Mn}^{++}$  on the activity of  $\phi\text{C2(W)}$  lysin

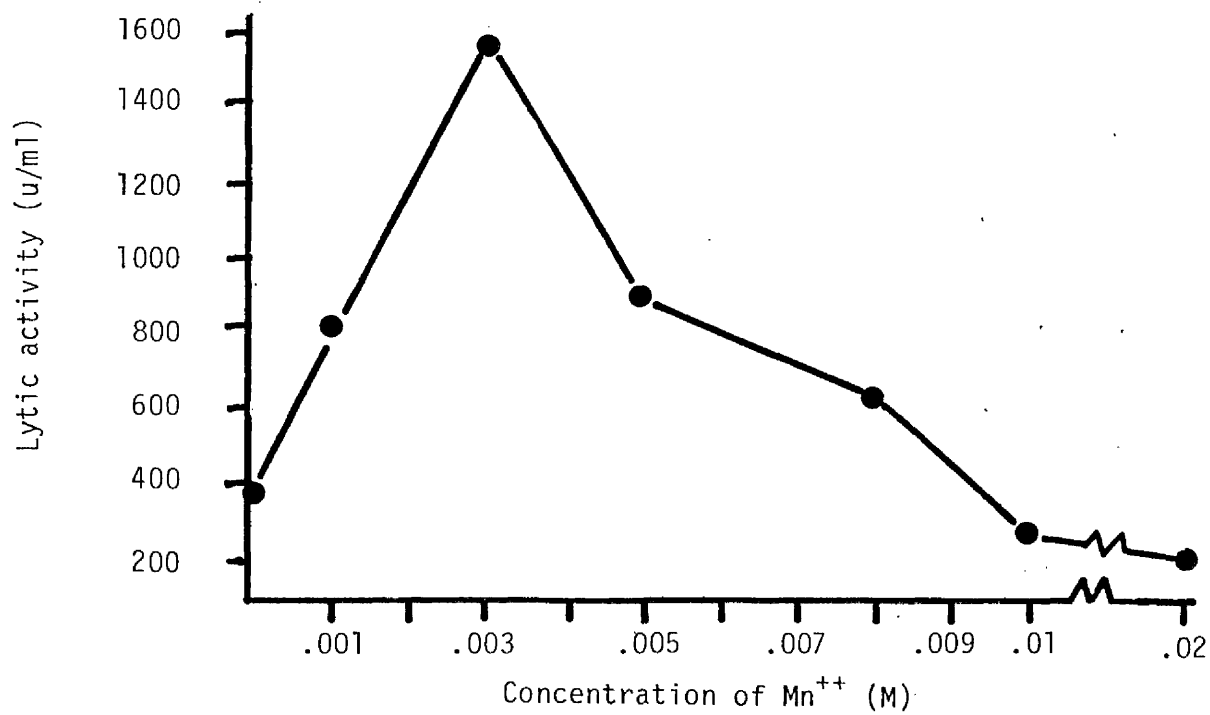
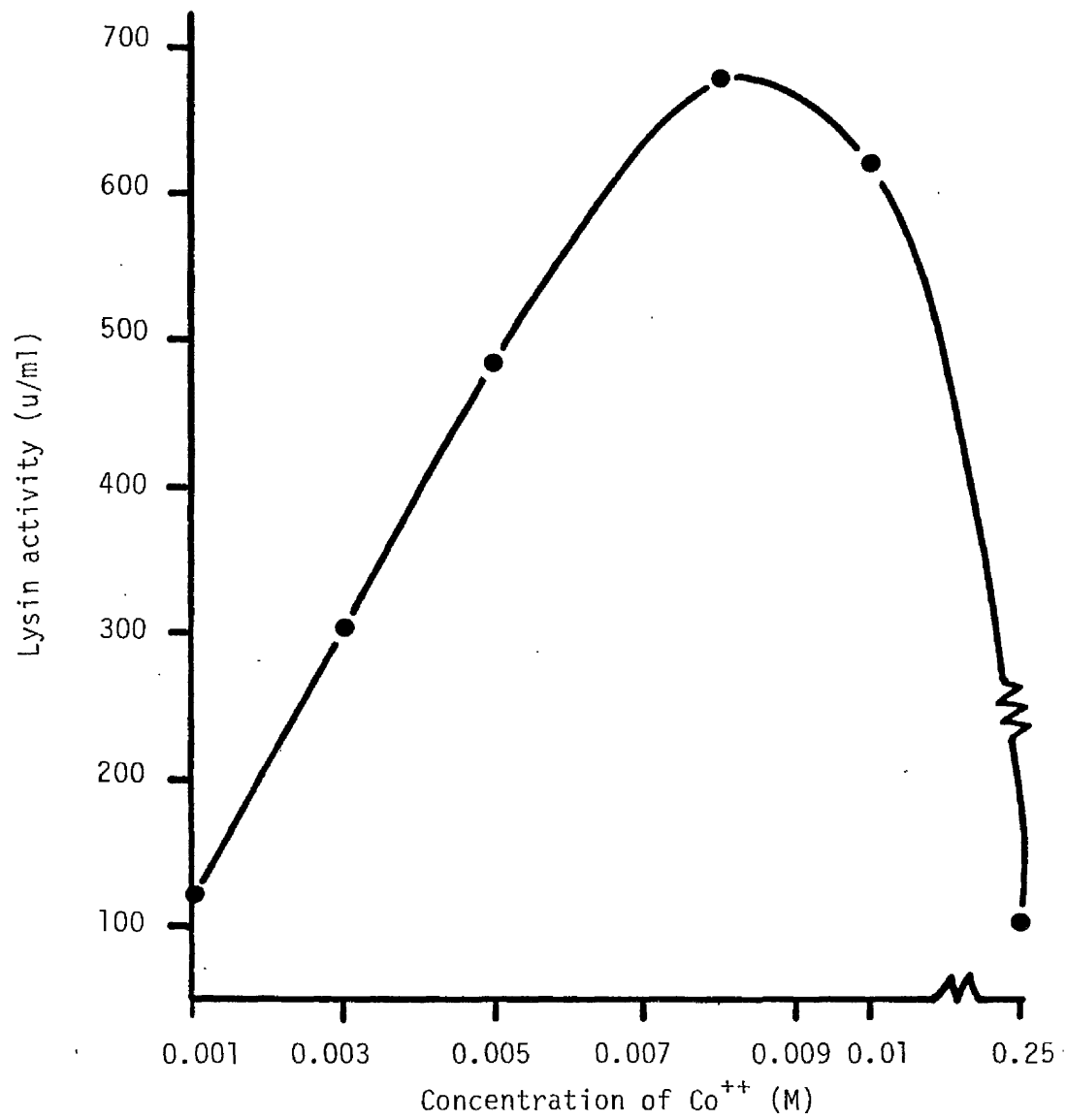


Figure 2.13 Effect of  $\text{Co}^{++}$  on the lytic activity of  $\phi\text{C2(W)}$  lysin





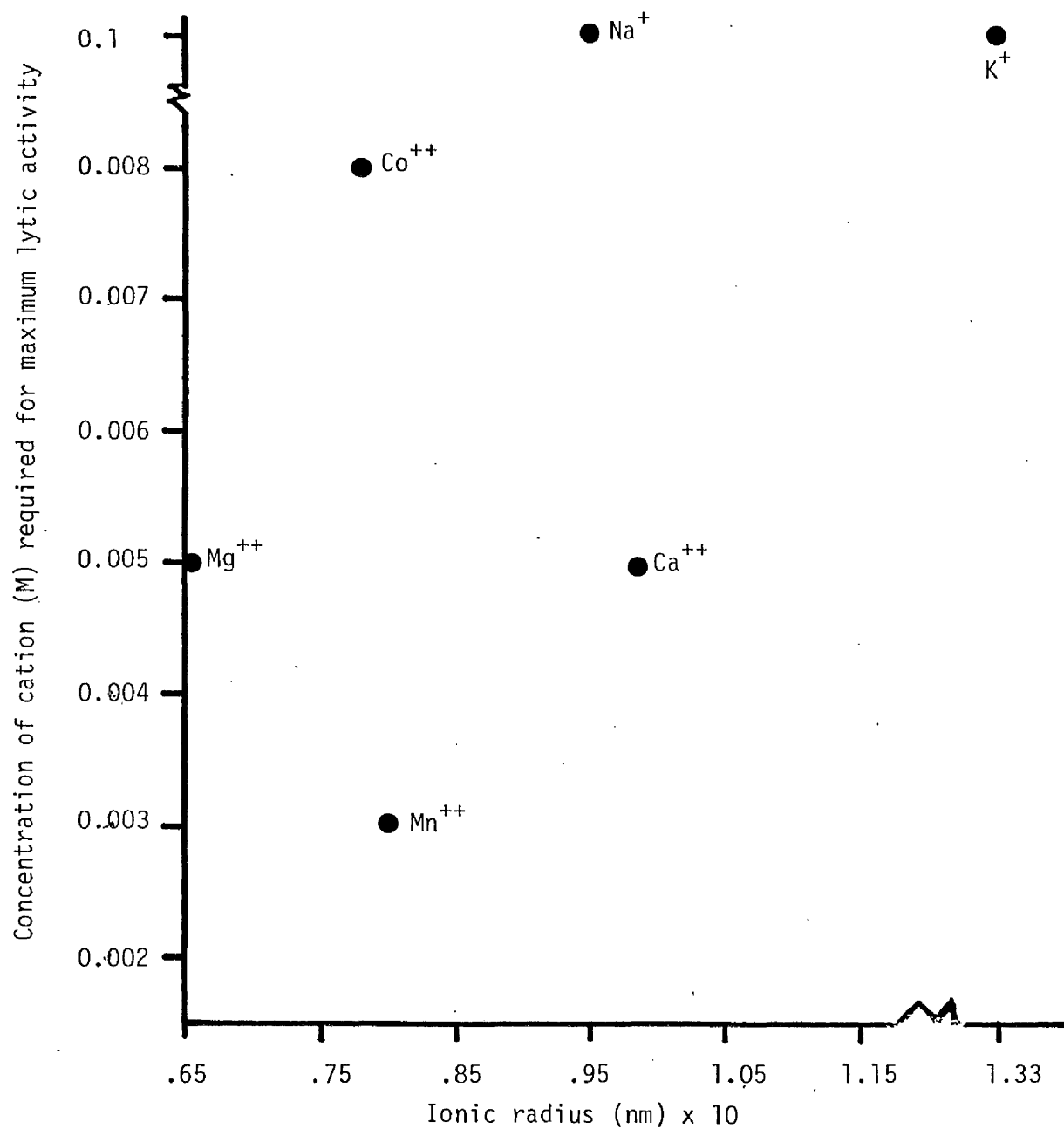
a broad response curve and an apparent concentration of 5 mM cation required for maximum lytic activity were found.

Since lower concentrations of divalent metal ions were required for optimum lytic activity compared with monovalent cations, an attempt was made to determine if there was a relationship between ionic size and the concentration of ion required for optimum lytic activity. A plot of ionic radius (Stark and Wallace, 1969) versus cation concentration is shown in Figure 2.14. The results for  $\text{Ca}^{++}$  and  $\text{Co}^{++}$  suggest that factors other than ionic size are also involved;  $\text{Co}^{++}$  is a much smaller ion than  $\text{Mn}^{++}$  yet higher concentrations of the former are required for maximum lytic activity. A similar situation exists for  $\text{Ca}^{++}$ ; this cation is larger than  $\text{Mg}^{++}$  yet similar ionic concentrations are required for maximum lytic activity with either ion. Nevertheless there was an apparent relationship between ionic radius and the concentration of  $\text{Mn}^{++}$ ,  $\text{Ca}^{++}$  and  $\text{K}^+$  required for maximum activity of  $\phi\text{C2(W)}$ -induced lysis. A similar relationship apparently existed for  $\text{Mg}^{++}$ ,  $\text{Co}^{++}$  and  $\text{Na}^+$ .

#### Effect of anions

The effect of 0.15 molar solutions of the following salts sodium chloride, sodium glutamate, sodium pyruvate, sodium citrate and sodium sulphate on the lytic activity of  $\phi\text{C2(W)}$  lysis was determined. No significant differences in lytic activity were found suggesting that in the case of  $\text{Na}^+$  salts, the anion has little influence on the lytic reaction.

Figure 2.14 Relationship between ionic radius and the concentration of cation required for maximum lytic activity of phage lysin



### Effect of inhibitors

Complete inhibition of the enzyme activity was obtained with a  $10^{-5}$  M solution of p-chloromercuribenzoate (Table 2.9). Complete reversal of the inhibitory action of a  $3.23 \times 10^{-4}$  M solution of the sulphydryl binding agent was obtained by the addition of 0.033 M cysteine. These results strongly suggest that  $\phi$ C2(W) lysin contains a sulphydryl group or groups which is/are essential for lytic activity. Attempts were made to obtain confirmation of the above data by attempting to inhibit the enzyme by oxidising the sulphydryl group(s). The most specific reagents for oxidising enzyme sulphydryl groups to disulphides are thought to be disulphides themselves e.g. oxidised glutathione (Dixon and Webb, 1964). The effect of oxidised glutathione on lytic activity is shown in Table 2.10. Unlike the situation with CMBA high concentrations ( $5.93 \times 10^{-2}$  M) of oxidised glutathione were required for complete inhibition. Marked inhibition of lytic activity was found in some but not all experiments with a 2.45 mM level of inhibitor. After these results were obtained mercaptoethanol was routinely added to the buffer used for elution in gel filtration experiments to maintain -SH groups of enzyme.

Hen egg lysozyme is inhibited by low concentrations of N-acetyl-d-glucosamine (NAG); a concentration of 28 mM of the amino sugar was reported necessary for 50% inhibition by Jolles *et al.* (1968). Concentrations of NAG in the range 0-50 mM did not inhibit the lytic activity of the phage enzyme.

Oram and Reiter (1965) reported that lysates of  $\phi$ ML3 contained a lysis inhibitor. The possibility that the basic substance isolated from PLGYG broth lysates (p123) could inhibit the  $\phi$ C2(W) lysin was studied.

Table 2.9

Effect of p-chloromercuribenzoate (CMBA) on the activity of  $\phi$ C2(W) lysin

Concentration of CMBA (M)	Lytic activity (u/ml)	% Inhibition
0	1200	0
$10^{-5}$	0	100
$10^{-6}$	325	73
$10^{-7}$	472	60.4
$10^{-8}$	1200	0

Table 2.10

Effect of oxidised glutathione on the  
activity of  $\phi$ C2 lysin

Concentration of oxidised glutathione (M)	Lysin activity (u/ml)
0	3583
$5.93 \times 10^{-2}$	0
$2.45 \times 10^{-2}$	250
$2.45 \times 10^{-3}$	3375
$2.45 \times 10^{-4}$	3500

Inhibitory effects could not be demonstrated using saturated solutions of the basic material. In these experiments freeze dried cells of C2 were suspended in 0.1 M potassium phosphate buffer saturated with the basic substance and used to titre lysin preparations.

Amphipathic substances such as lipoteichoic acids, cardiolipin and dipalmitoyl phosphatidyl glycerol have been shown to inhibit the activity of some autolytic enzymes (Cleveland *et al.*, 1975; Cleveland *et al.*, 1976; Rogers, Perkins and Ward, 1980; Shungu, Cornett and Shockman, 1980). Cardiolipin (diphosphatidylglycerol) has been reported to be a particularly potent inhibitor of the autolytic N-acetylmuramoyl-hydrolase (muramidase) of *Str. faecalis* ATCC9790 (Shungu *et al.*, 1980). The effect of cardiolipin on the activity of  $\phi$ C2(W) lysis was determined. Concentrations of cardiolipin in the range 0.0098 mM - 0.098 mM had no significant effect on the activity of the phage enzyme.

Almost complete inhibition of lytic activity was found with 1 M urea. Rivanol (6-9 diamino - 2-ethoxyacridine) was used in the partial purification of T4 phage lysozyme by Tsugita *et al.* (1968) to precipitate DNA and acidic proteins. The lytic activity of the  $\phi$ C2(W)-induced lysin was completely inhibited by concentrations required for DNA and acidic protein precipitation (0.25% w/v).

During the course of the work a wide range of compounds was tested for their effect on the lytic activity of  $\phi$ C2(W) lysin. Because it is sometimes important to have data concerning substances which do not interfere with enzyme reactions a list of substances found not to significantly affect the action of the lysin is given. The highest concentration tested is given in parenthesis. The compounds tested were: n-acetylmuramic acid (10 mM); cysteine (33 mM); glucose (28 mM); ethyldiaminetetracetic acid (5 mM); 2 mercaptoethanol (10 mM);

l-glycine (13.3 mM); D/L alanine (11.2 mM); galactose (5.5 mM); salicin (3.5 mM); maltose (2.9 mM); melibiose (2.9 mM); l-rhamnose (6.1 mM); cellobiose (2.9 mM); raffinose (2.0 mM); sodium azide (30.8 mM); streptomycin sulphate (3.7 mM); glycylglycine (7.6 mM); l-arginine (5.7 mM); l-glutamine (6.9 mM); penicillin G (5 IU/ml) and potassium thiocyanate (0.09 M). The latter compound is a chaotropic agent which is useful in eluting substances strongly bound to column packing materials.

#### Molecular weight of the $\phi$ C2(W)-induced lysin

A 90 x 2.6 cm column of Bio-Gel P100 was calibrated with a mixture of protein standards. Providing that the flow rate did not exceed about 15 ml h<sup>-1</sup> (a linear flow rate of 2.83 ml cm<sup>-2</sup> h<sup>-1</sup>) good resolution of the standards was obtained (Figure 2.15). At higher flow rates it was more difficult to obtain good resolution of bovine serum albumin and ovalbumin. The results of three calibration experiments were meaned and used to derive the regression equation used to construct the calibration curve shown in Figure 2.16. For comparison the elution volumes found for each standard are also shown. The line was defined by the equation  $Y = -177.3592X + 990.8134$ , where  $Y$  = elution volume in ml and  $X$  = log molecular weight of the protein standard. The correlation coefficient was 0.992. The only protein standard which was out of line was ovalbumin whose chromatographic behaviour was consistent with a globular protein of molecular weight about 48,000 instead of its reported value of 43,000 given in the Pharmacia gel filtration calibration kit instruction manual. Substitution of the elution volume of the phage lysin into the regression equation gave a mean value (three determinations) of  $46,282 \pm$  a standard deviation of 269 for the molecular weight of the phage enzyme.

Figure 2.15      Chromatography of molecular weight markers on a 90 cm x 2.6 cm column of Bio-Gel P100

— 0.D.<sub>280</sub>

Column operating conditions

Sample volume, 4.5 ml

Eluant, 0.2 M potassium phosphate, pH 7.3

+ 0.01% (w/v)  $\text{NaN}_3$

Flow rate, 15.05 ml h<sup>-1</sup>

Fraction volume, 3.763 ml

Temperature, 5°C

Molecular weight markers

A - bovine serum albumin

B - ovalbumin

C - chymotrypsinogen A

D - Ribonuclease



Figure 2.15

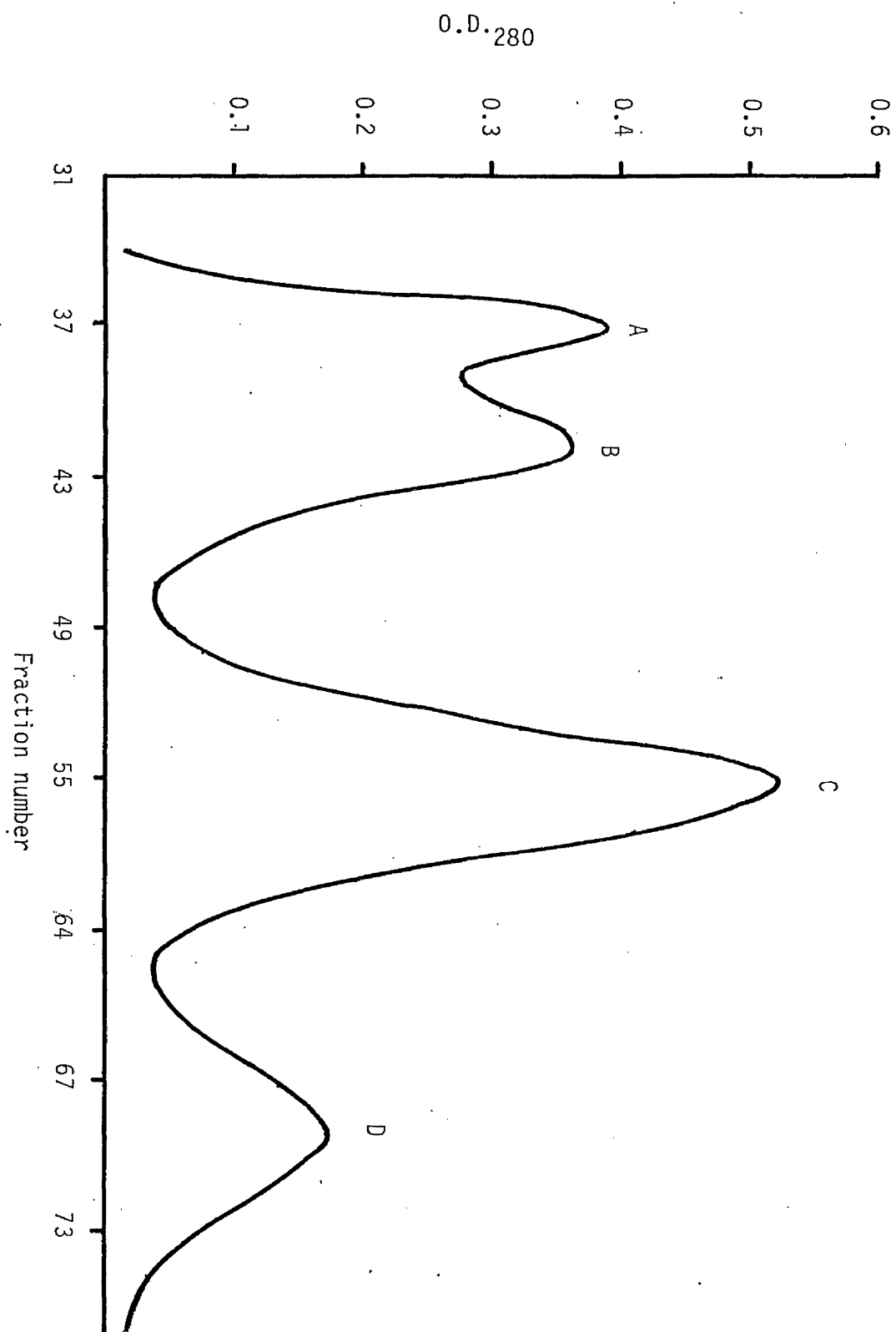


Figure 2.16 Elution volume ( $V_e$ ) versus log molecular weight calibration curve for the Bio-Gel P100 column

Operating conditions are defined in Figure 2.15. The line was obtained by averaging the results of three experiments and subjecting the mean values to regression analysis.

—●—, results of experiment 1

—○—, results of experiment 2

—□—, results of experiment 3

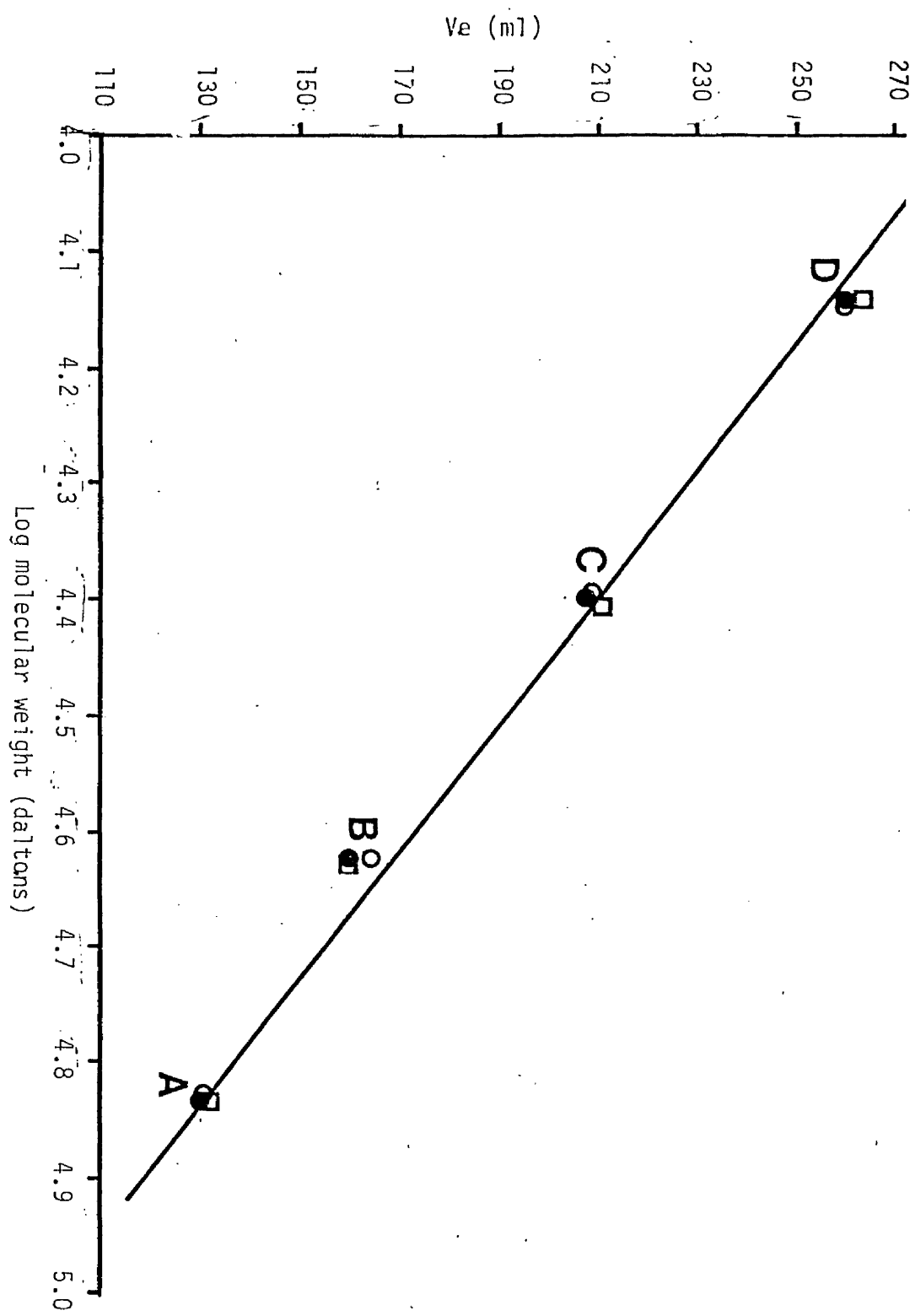
A, bovine serum albumin

B, ovalbumin

C, chymotrypsinogen A

D, ribonuclease

Figure 2.16



### Sensitivity of lactic acid bacteria and selected bacteria to $\phi$ C2(W)-induced lysin

Logarithmic phase cultures of the test bacteria were harvested, washed, suspended in buffer and their sensitivity to lysin determined (Table 2.11). All strains of the three species of Group N streptococci tested were lysed. Marked strain dependant differences in sensitivity, however, were apparent. *Str. cremoris* strains R6,1249 and *Str. lactis* C10 were particularly sensitive whereas *Str. cremoris* strains US3, AM2 and *Str. diacetylactis* DRC2 were relatively insensitive. The differences in sensitivity are perhaps more apparent when lytic activities are expressed relative to the activity towards a reference strain (freeze-dried cells of C2) as suggested by Oram and Reiter (1965). That commercial freeze-dried starters (containing a mixture of undefined strains of Group N streptococci) intended for bulk starter inoculation were also lysed is apparent.

All strains of the four species of Group D streptococci tested were lysed. Strain-dependent differences in sensitivity were also apparent.

Strains of *Str. dysgalactiae*, *Str. thermophilus*, *Lact. bulgaricus*, *Lact. fermenti*; *Leuc. lactis*, *Leuc. dextranicum*, *Leuc. cremoris*, *E. coli* and *Micrococcus lysodeikticus* were not lysed.

### Relationship between the adsorption of $\phi$ C2(W) to Group N streptococci and sensitivity to $\phi$ C2(W) lysin

In a preliminary report, Reiter and Oram (1963) stated that phage and lysin competed for the same adsorption site;  $\phi$ ML3 lysin could be inhibited by the homologous phage. An attempt was made to determine whether there was a relationship between the ability of a strain to adsorb  $\phi$ C2(W) and its susceptibility to lysin. The sensitivity of

Table 2.11

Sensitivity of lactic acid bacteria to  $\phi$ C2(W) lysin

Culture	Strain identity	Activity ( $\mu$ ml <sup>-1</sup> )	Relative activity <sup>(2)</sup>
<i>Str. cremoris</i>	E8	15,928	193
	SK11	5,227	63
	SC1	10,087	122
	HP	5,227	63
	1249	16,506	200
	AM2	260	3
	US3	275	3
	R6	15,818	192
<i>Str. lactis</i>	RM1	6,602	78
	ML8	8,253	100
	C2 <sup>(1)</sup>	8,253	100
	ML3	3,301	40
	C10	24,759	300
	WM1	7,565	92
<i>Str. diacetylactis</i>	DRC2	344	4
	1246	4,127	50
<i>Str. thermophilus</i>	BC	0	
	NIRD7	0	
<i>Str. durans</i>	NCD0498	2,400	29
	NCD0596	800	10
<i>Str. bovis</i>	NCD0597	1,800	22
	NCD0598	500	6
<i>Str. faecium</i>	NCD0942	800	10
<i>Str. faecalis</i>	NCD0581	500	6
<i>Str. dysgalactiae</i>	NCD01356	0	
	NCD01357	0	
<i>Leuc. cremoris</i>	NCD0543	0	
	NCD0705	0	
<i>Leuc. dextranicum</i>	NCD0516	0	
	NCD0861	0	
<i>Leuc. lactis</i>	NCD0532	0	
	NCD0549	0	
Commercial concentrate	A <sup>(3)</sup>	2,570	31
Commercial concentrate	B <sup>(3)</sup>	1,285	16

A purified  $\phi$ C2(W) lysin preparation was assayed against the test cultures as described in Methods.

(1) Freeze-dried C2 cells.

(2) Relative activity  $\frac{\text{Activity towards strain} \times 100}{\text{Activity with freeze-dried C2 cells}}$

(3) Preparation of freeze-dried lactic streptococci normally used for bulk starter inoculation.

several streptococci to lysin and their capacity to adsorb  $\phi C2(W)$  is shown in Table 2.12. No adsorption of  $\phi C2(W)$  to SC1 or DRC2 was found. Since DRC2 was relatively insensitive to the phage lysin and SC1 was very sensitive to lysin these findings suggest that lysin and phage have different sites of action. This supposition is supported by data for the other strains e.g. an absorption figure of 37% was found for strain 1249 which had a relative sensitivity of 200. Comparison of the data for ML3 shows that a high adsorption of phage occurred yet this strain had a considerably lower sensitivity to lysin.

#### Ultrastructural studies of the action of phage lysin on *Str. lactis* C10

The action of  $\phi C2(W)$  lysin on C10 cells suspended in 0.1 M k-phosphate buffer at 37°C was studied by electron microscopy.

The cells were incubated with 300 u/ml of lysin. Samples were removed at intervals and the reaction stopped by fixing the cells in PBS + glutaraldehyde as described in "Methods". The cells were then embedded in resin and sectioned. Control cells had clearly defined cell walls but the cell membrane was difficult to see clearly (Plate 2.1). After 30 sec (Plate 2.2) the cells containing 300 u/ml of lysin had lost most of their cell wall material and distortion of shape was apparent. Pieces of cell wall material appeared to have been removed from some cell walls and could be seen to be attached like strings to some of the cells. After 150 sec (Plate 2.3) extensive lysis was apparent. Vacuolation was also visible. Protoplasts were clearly visible. Examination of over 30 fields of view showed virtually no cell wall material on cells at this time. Occasionally an apparently unaffected coccus was found. A micrograph (Plate 2.4) taken at higher magnification during the course of the reaction shows C10 cells with virtually all the cell wall material absent. Some material, probably partially digested

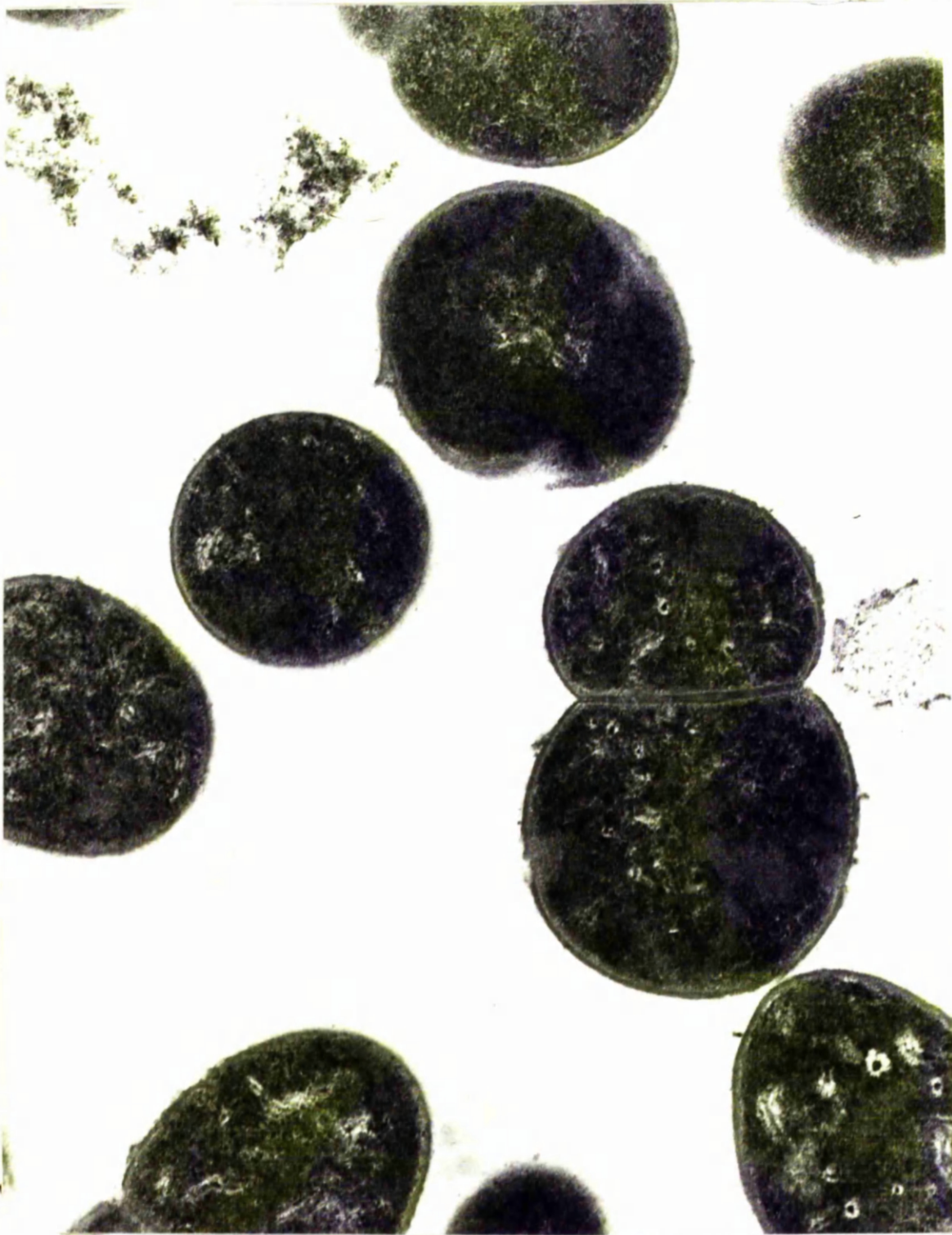
Table 2.12

Comparison of  $\phi$ C2(W) phage adsorption and strain  
sensitivity to  $\phi$ C2(W) lysin

Strain	% Adsorption	Relative sensitivity to lysin
C2	99	100
ML3	83	40
E8	14	193
ML8	27	100
DRC2	0	4
C10	54	300
1249	37	200
SC1	0	122

$$\text{Relative sensitivity} = \frac{\text{Activity of } \phi\text{C2 lysin towards strain} \times 100}{\text{Activity with freeze-dried C2 cells}}$$

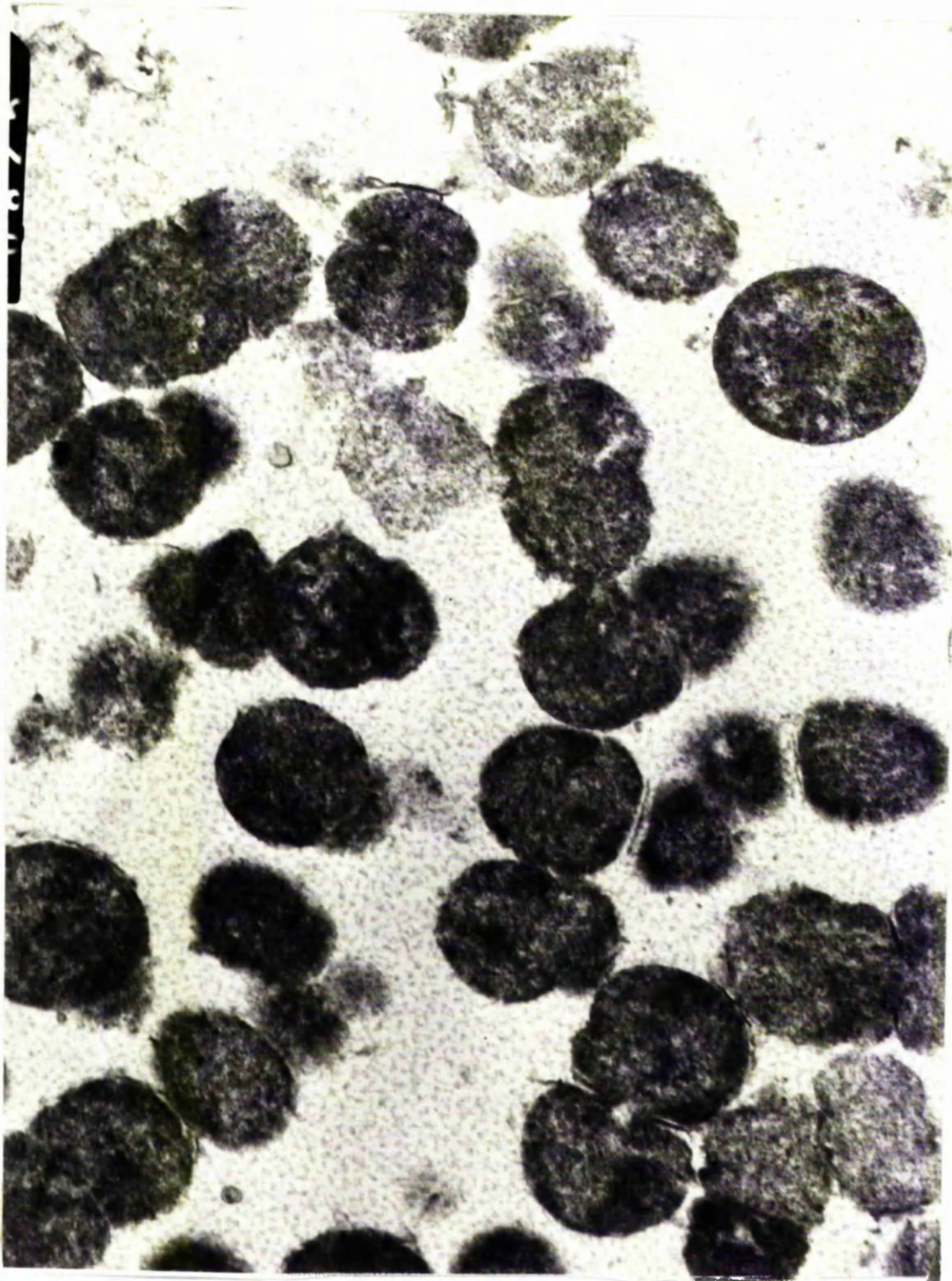
Plate 2.1      Electronmicrograph showing the effect of phage lysin on  
cells of *Str. lactis* C10



No phage lysin added.    x 40,000



Plate 2.2 Electronmicrograph showing the effect of phage lysin on cells of *Str. lactis* C10

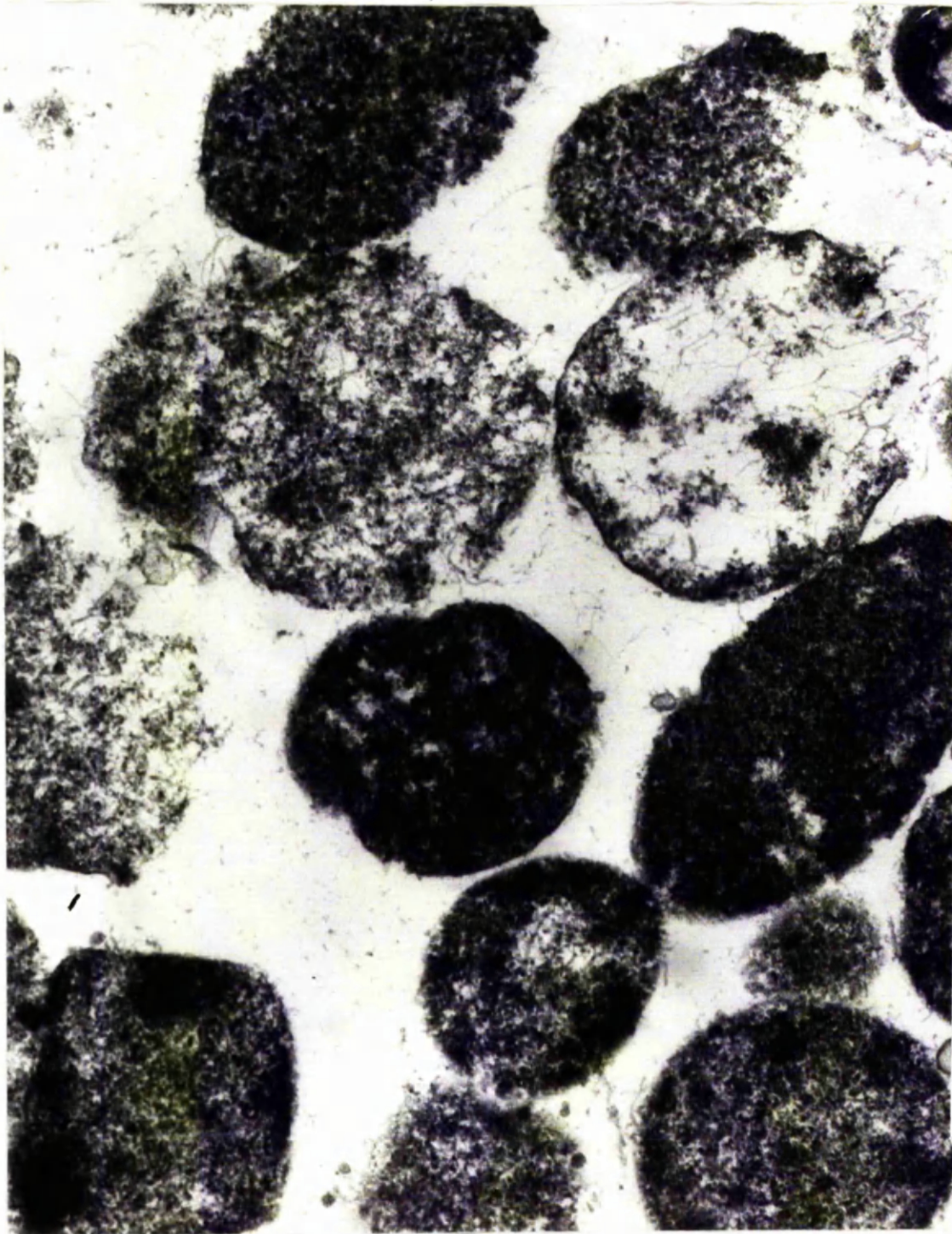


Cells were incubated with 300 u/ml of lysin for 30 s at 37°C.

x 23,000



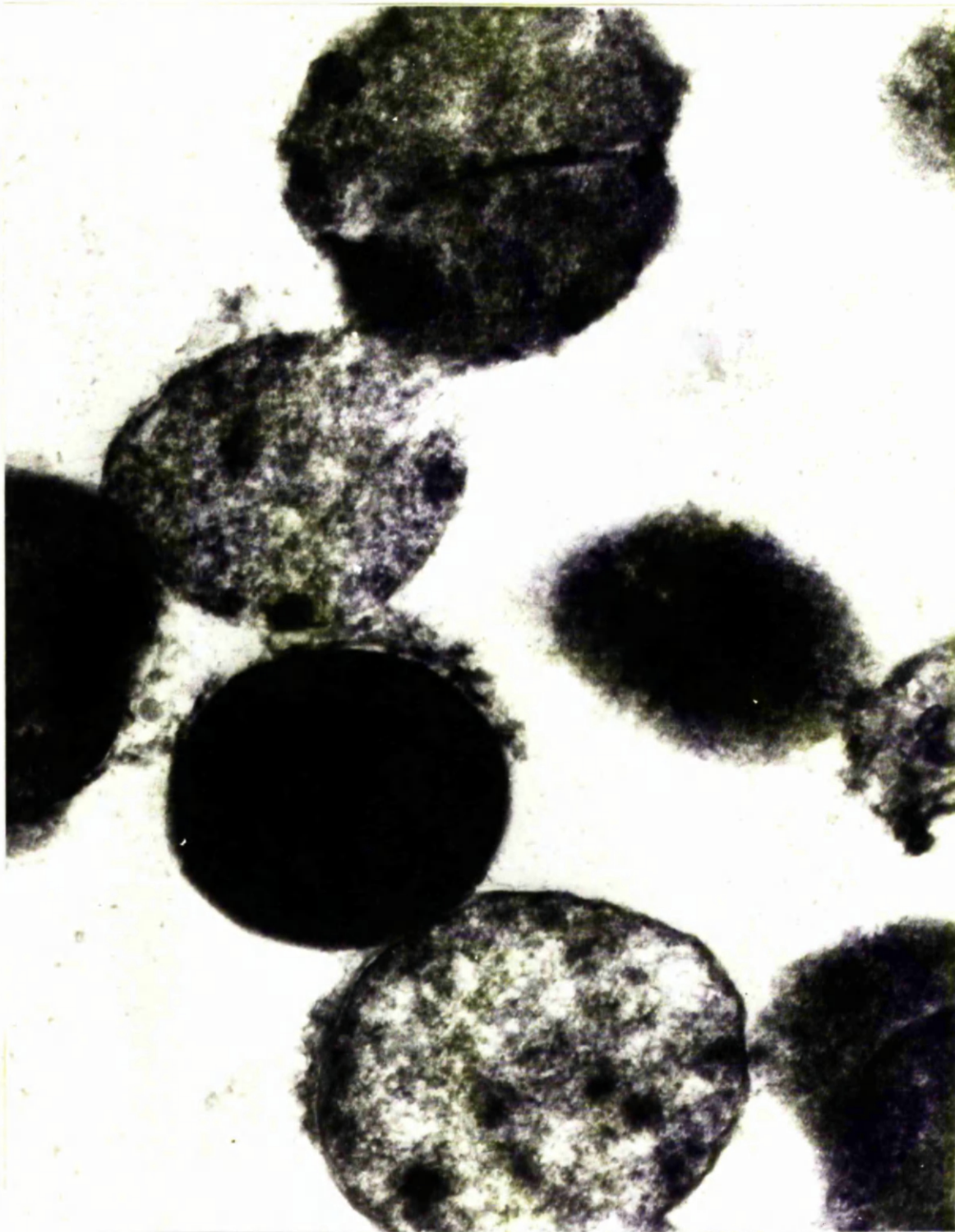
Plate 2.3 Electronmicrograph showing the effect of phage lysin on cells of *Str. lactis* C10



Cells were incubated with 300 u/ml of lysin for 150 s at 37°C.

x 40,000

Plate 2.4 Electronmicrograph showing the effect of phage lysis on cells of *Str. lactis* C10



Cells were incubated with 300 u/ml of lysis for 150 s at 37°C.

x 50,000

cell wall material, is also clearly visible. These results show clearly that the cell wall is the site of action of the  $\phi$ C2(W)-induced lysin and that lysin action removes the cell wall creating protoplasts.

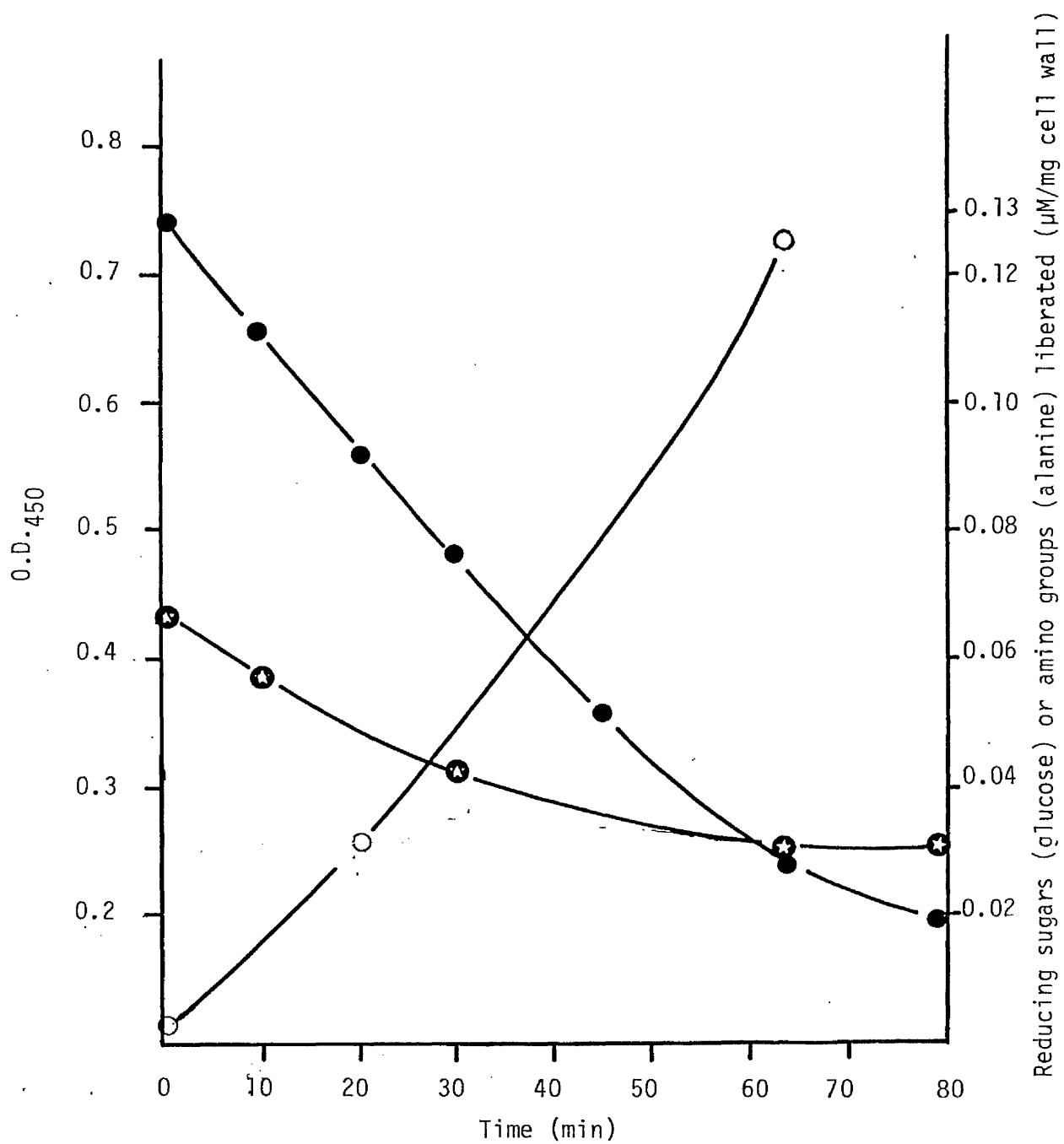
#### Identification of cell wall linkage attacked by $\phi$ C2(W) lysin

The cell wall of Gram-positive bacteria consists almost entirely of a heteropolymer called peptidoglycan or murein. This polymer contains chains of amino sugars cross linked by peptides. To determine whether the lytic enzyme was a glycosidase which hydrolyses linkages between alternating amino sugars releasing reducing sugars, or an amidase which attacks the linkage between the amino sugar and peptide chains liberating free amino groups, or an endopeptidase which attacks the peptide chains also producing free amino groups, cell walls were incubated with enzyme and samples were assayed to identify the chemical groups liberated. The results obtained (Figure 2.17) reveal that lysis of cell walls is accompanied by the release of reducing sugars. The release of these compounds approximately parallels cell wall lysis. No amino group release was detected. These results clearly show that the  $\phi$ C2(W)-induced enzyme is a glycosidase. It is of interest to note that the rate of C10 wall lysis occurred at a considerably reduced rate compared with that of entire cells of C10. If C10 cells, adjusted to a similar turbidity, were used in place of walls, almost complete lysis had occurred after 5 min incubation.

To determine whether the glycosidase was a muramidase which hydrolyses the n-acetylmuramyl-N-acetylglucosamine linkage or a glucosaminidase which cleaves the N-acetylglucosaminy-N-acetylmuramic linkage, the cell wall digest was reduced to convert the free end group released to the corresponding alcohol, and analysed for amino sugars and their derivatives using an amino acid analyser. Before presenting



Figure 2.17 Action of partially purified  $\phi$ C2(W) lysin on cell walls of *Str. lactis* C10



- , turbidity of cell walls incubated with  $\phi$ C2(W) lysin in buffer at 37°C
- , reducing sugars liberated
- ★—, amino groups liberated

the results it is appropriate to comment on the chromatographic behaviour of standard amino sugars and their derivatives. The chromatograph presented in Figure 2.18 is a composite and was prepared from the chromatographs of individual compounds. It is apparent that the amino sugars and their reduced derivatives were well resolved. It is also noteworthy that muramicitol gave an extremely low colour response compared with the other standards. Because peptidoglycan also contains amino acids, samples of amino acids were also chromatographed with some amino sugar standard solutions. The retention times of some cell wall components and their derivatives are shown in Table 2.13. Although glutamic acid and muramic acid and muramicitol and aspartic acid were resolved the retention times of each pair were similar.

Chromatography of the hydrolysed, reduced wall lysates (Figures 2.19 and 2.20) produced using method A (p113) revealed that glucosamine levels were similar in control and enzyme treated cells showing that the glucosamine linkage was not attacked. This was confirmed by the failure to detect the formation of glucosaminitol. Muramic acid was not clearly detected in the hydrolysates of either the control or enzyme treated lysate. However, a 'shoulder' was apparent on the glutamic acid peak of the control lysate suggesting that muramic acid was present.

Virtually no trace of 'muramic acid' was found in the enzyme treated walls and no evidence for muramicitol was found in the control cell walls. However a hump was apparent at the start of the aspartic acid peak (Figure 2.20) where muramicitol would be expected suggesting that muramicitol was present in the reduced, enzyme treated, cell wall digest. Further attempts to identify the linkage attacked using higher concentrations of cell walls did not yield more definitive data.

Figure 2.18      Chromatographic behaviour of amino sugars and their derivatives  
on the J. L. amino acid analyser

Chromatogram is a composite prepared from the results obtained from the chromatography of individual samples.

The concentration of compounds applied was as follows: N-acetyl-D-muramic acid, 0.21  $\mu$ M; N-acetyl-D-glucosamine, 0.16  $\mu$ M; muramicitol, 2.1  $\mu$ M and glucosamicitol, 1.6  $\mu$ M.

Mur,      muramic acid

Gla,      glucosamine

Mur-OH, muramicitol

Gla-OH, glucosamicitol

Figure 2.18

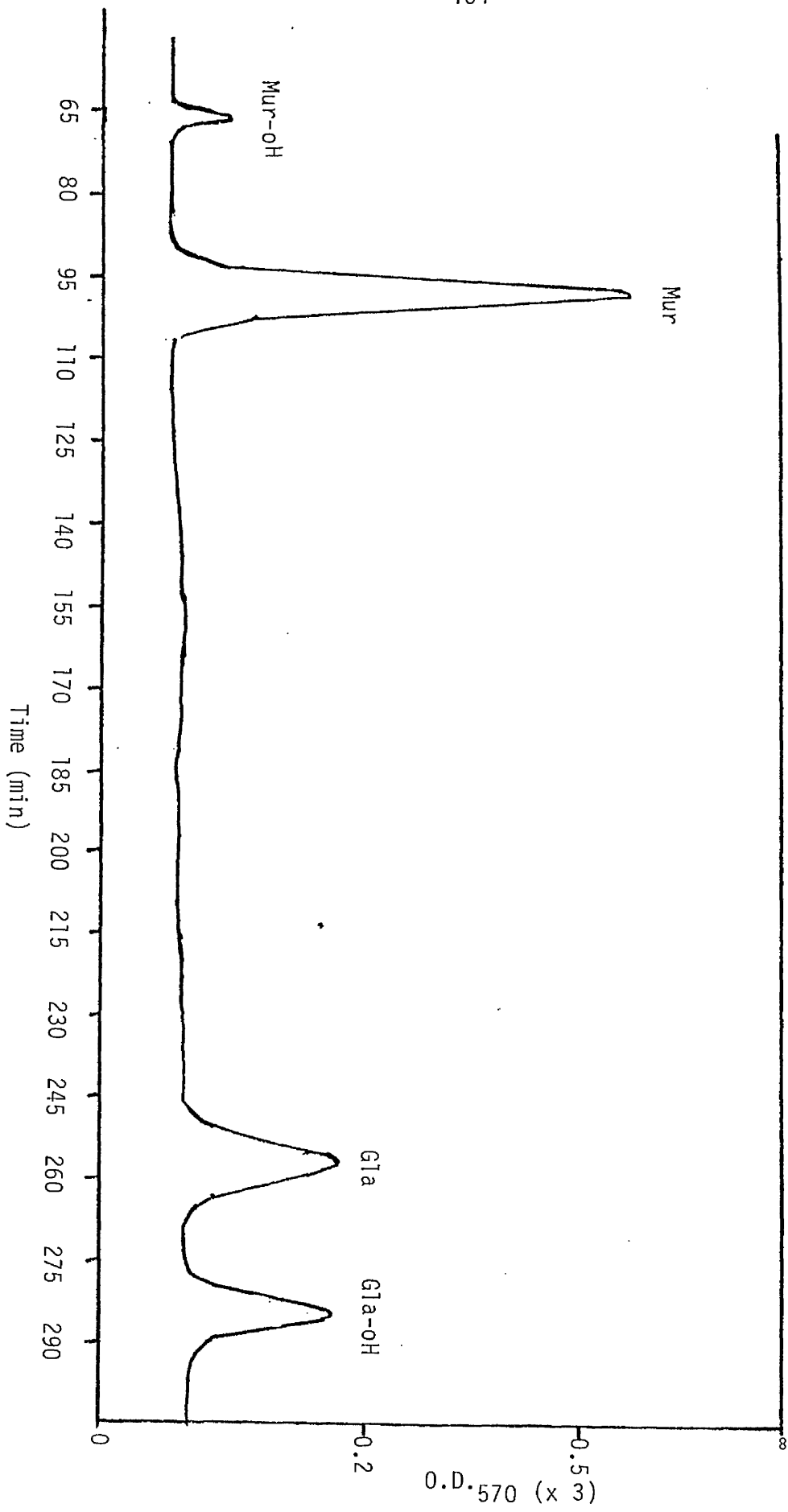




Table 2.13

Retention times of some cell wall components and their derivatives  
on the J. L. Amino Acid Analyser

Compound	Retention time (min)
muramicitol	65
aspartic acid	67.5
muramic acid	94
glutamic acid	97.5
glucosamine	252.5
glucosamicitol	279.3

The amino acid analyser was operated under the normal conditions for amino acid analysis except that a temperature of 45°C was used and the buffer was changed from pH 3.25 to pH 4.25 at 110 min.

Figure 2.19 Chromatogram showing amino sugars, amino sugar derivatives and amino acids in cell walls of *Str. lactis* C10

Cell walls were hydrolysed using Method A in Methods. Concentration of cell walls used for analysis was equivalent to 1.0 mg/ml

Asp, aspartic acid

Glu, glutamic acid

Ala, alanine

Gla, glucosamine

Figure 2.19

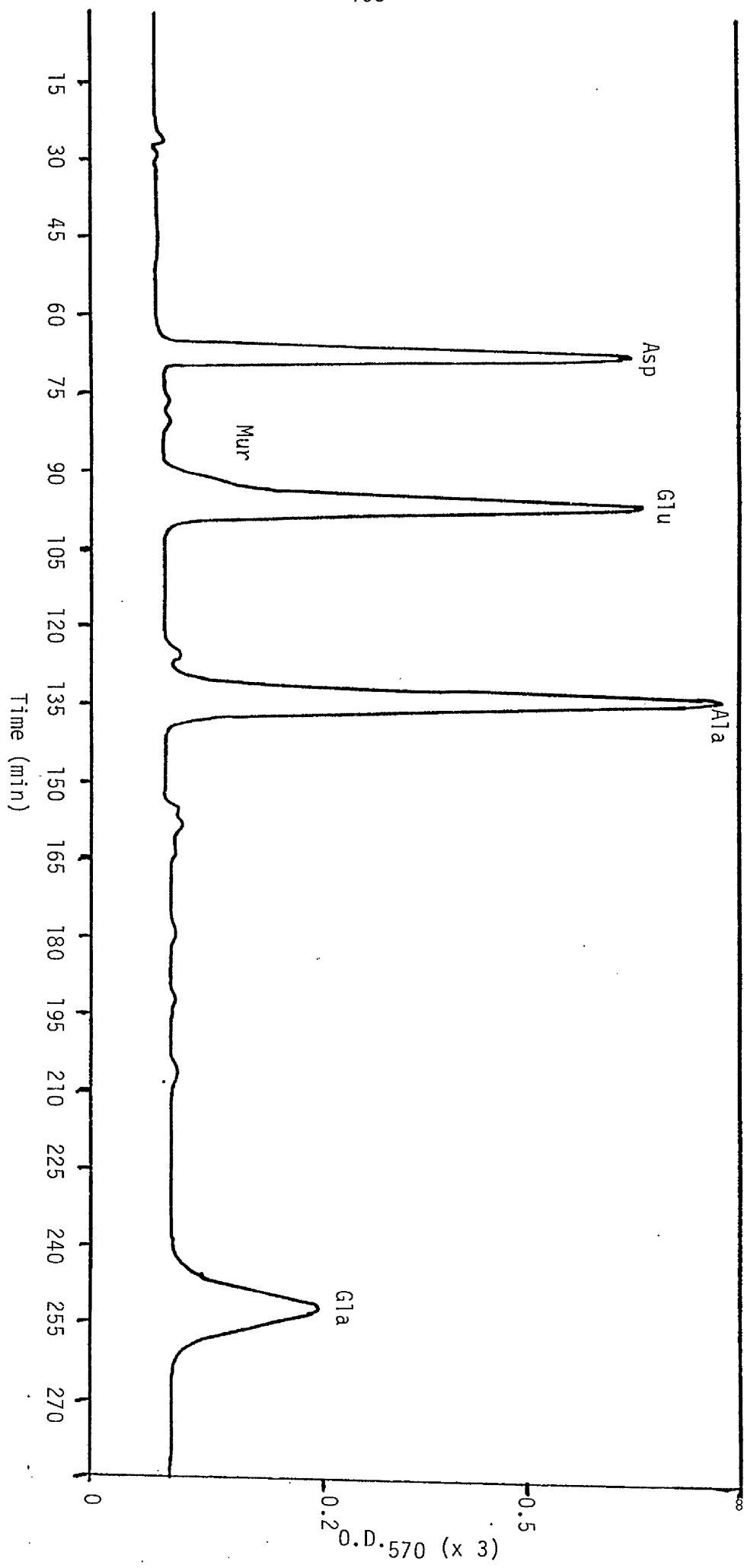
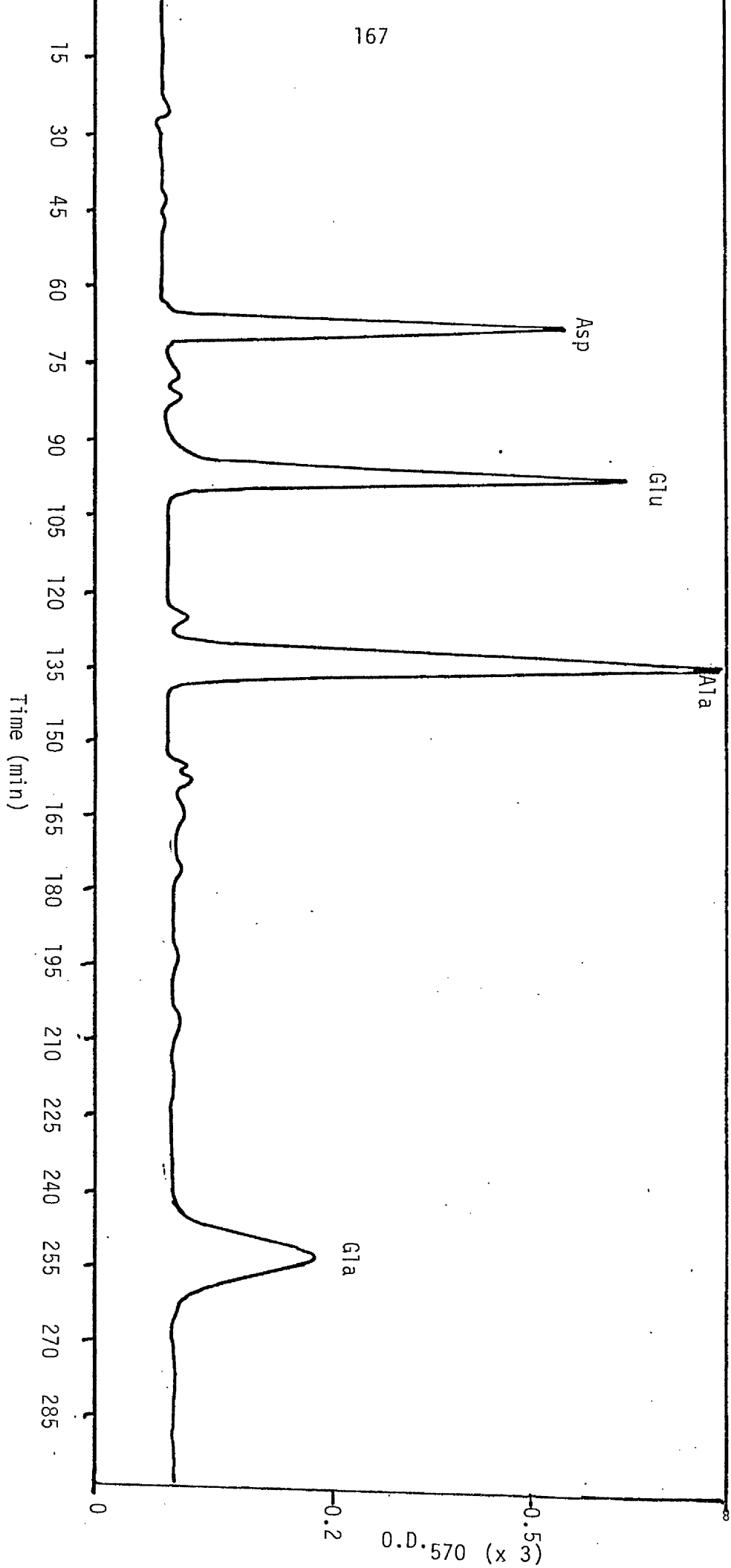


Figure 2.20      Chromatogram showing amino sugars, amino sugar derivatives and amino acids in cell walls of *Str. lactis* C10 incubated with  $\phi$ C2(W)-induced lysin

Cells walls were hydrolysed using Method A in Methods. Concentration of cell walls used was 1.0 mg/ml.

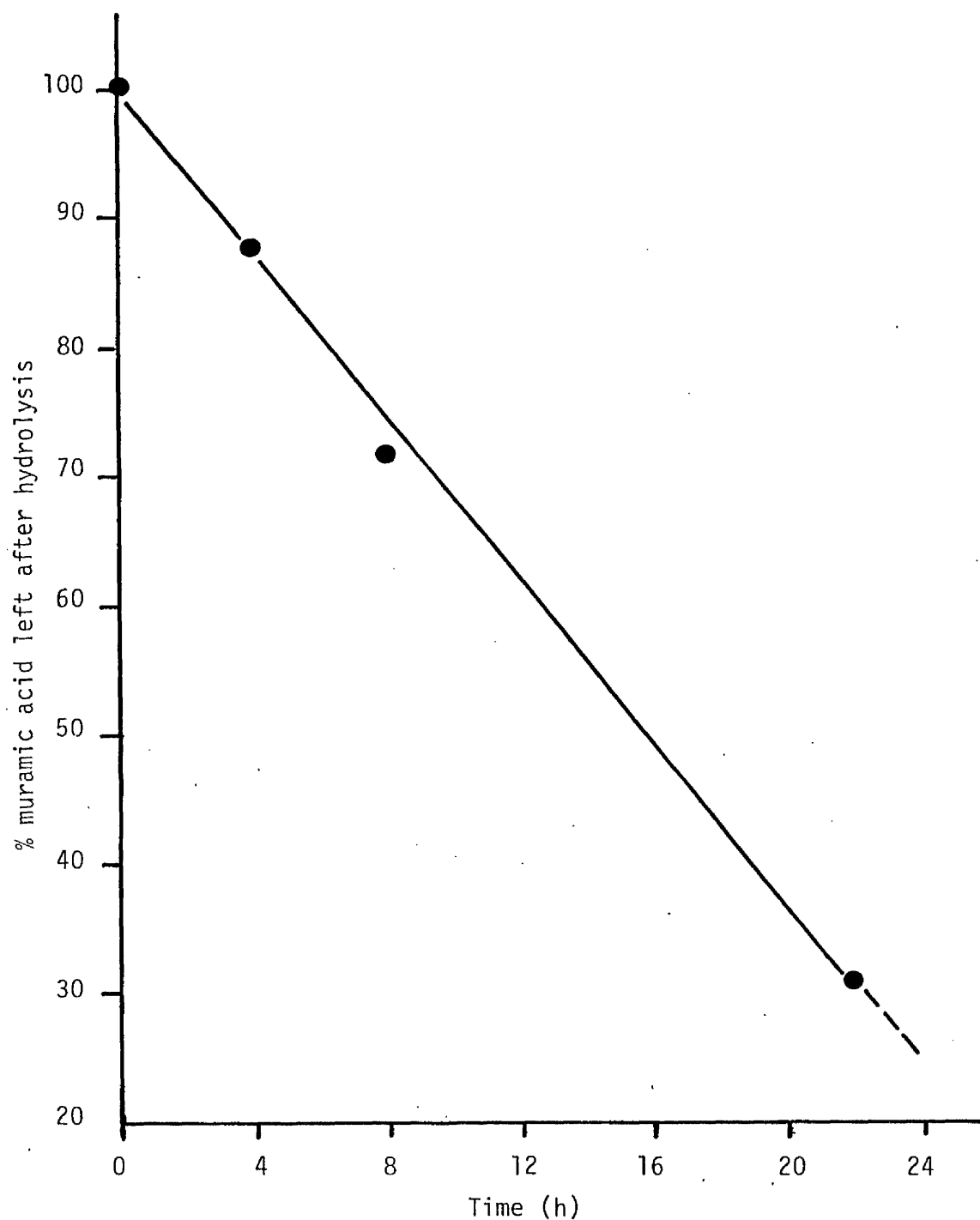
Figure 2.20



The fact that muramicitol could not be clearly detected in the previous experiment was not totally unexpected in view of the poor colour response of the standard (see Figure 2.18). That muramic acid was not clearly identifiable was unexpected since Tsugita *et al.* (1968) who used a similar methodology did not report difficulties in the determination of this amino sugar. Since muramic acid is a fairly unstable compound the possibility that muramic acid was degraded during hydrolysis was considered. The results of experiments in which muramic acid was held in 6 M acid at 110°C for various times (Figure 2.21) showed that muramic acid was unstable under the hydrolysis conditions used in the previous experiment. These results suggested that it might be possible to demonstrate more clearly the presence of muramic acid by using less severe hydrolysis conditions than those used previously.

Chromatography of reduced enzyme treated and control cell walls hydrolysed by method C (p113) revealed the presence of muramic acid in control cell walls (Figure 2.22) and only a slight trace of this amino compound in enzyme treated cell walls (Figure 2.23). Definitive evidence that  $\phi$ C2(W) lysin was a n-acetylmuramidase requires the disappearance of muramic acid to be balanced by the production of the reduced product, muramicitol. Unfortunately it was not possible to determine whether muramicitol was produced because an unidentified substance(s) was/were present in the position where the alcohol would be expected in both control and enzyme treated cell walls. This material was probably a peptide or peptides which would have been hydrolysed if more extreme hydrolysis conditions had been used. As in the previous experiment no evidence for a reduction in the glucosamine content of enzyme treated cells nor the production of glucosamicitol was found.

Figure 2.21 Effect of holding muramic acid in 6 M hydrochloric acid at 110°C on the concentration of muramic acid remaining



Samples of N-acetyl-muramic acid (0.8 mg) were hydrolysed in 6 M HCl and analysed for muramic acid at the times shown using the amino acid analyser. The peak area of each sample was calculated and is expressed as a percentage of the zero time peak volume to give the percentage muramic acid remaining

Figure 2.22      Amino sugars, amino acids and amino sugar derivatives in cell walls of *Str. lactis* C10

Cell walls were hydrolysed using Method C in Methods. Concentration of cell walls used was 0.53 mg/ml.



Figure 2.22

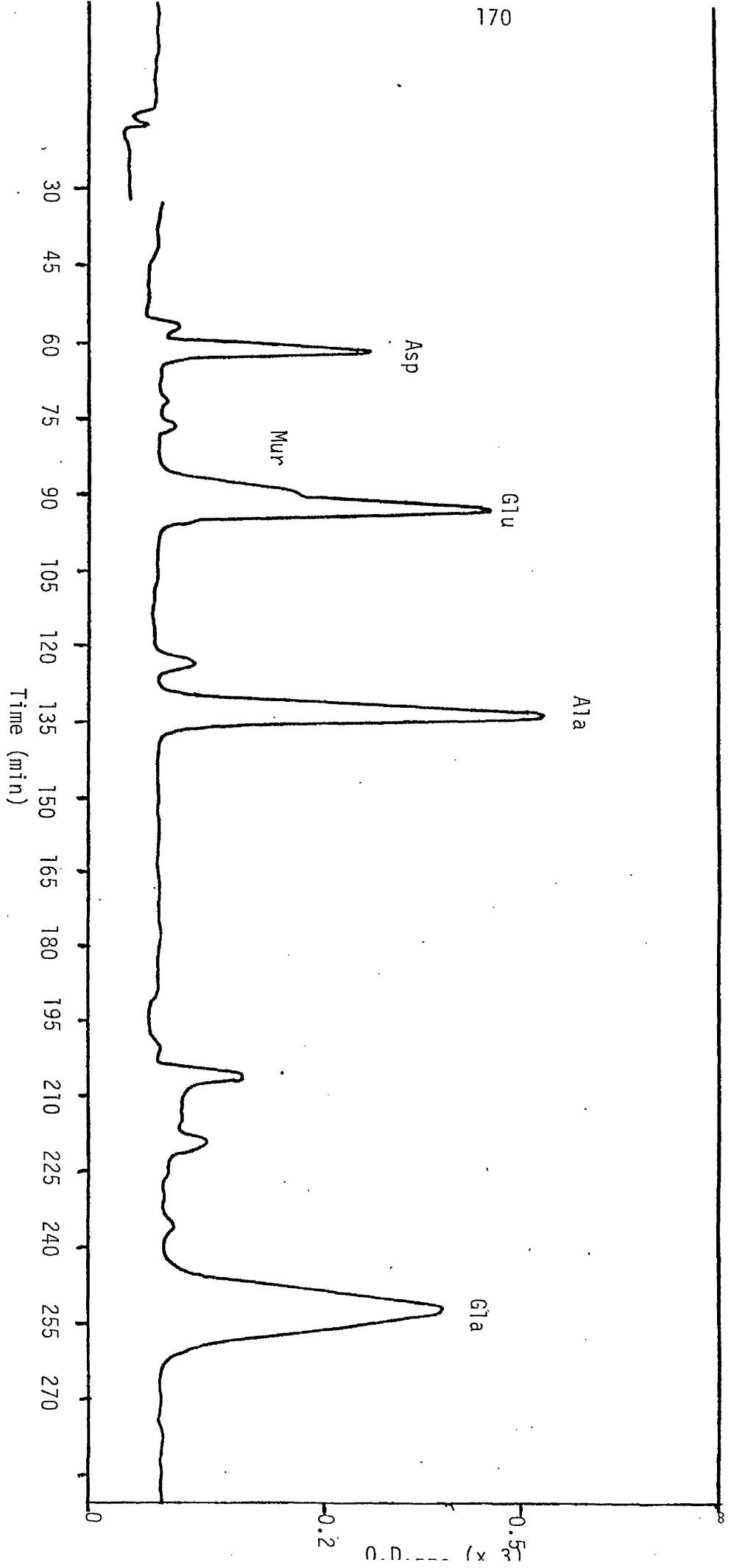
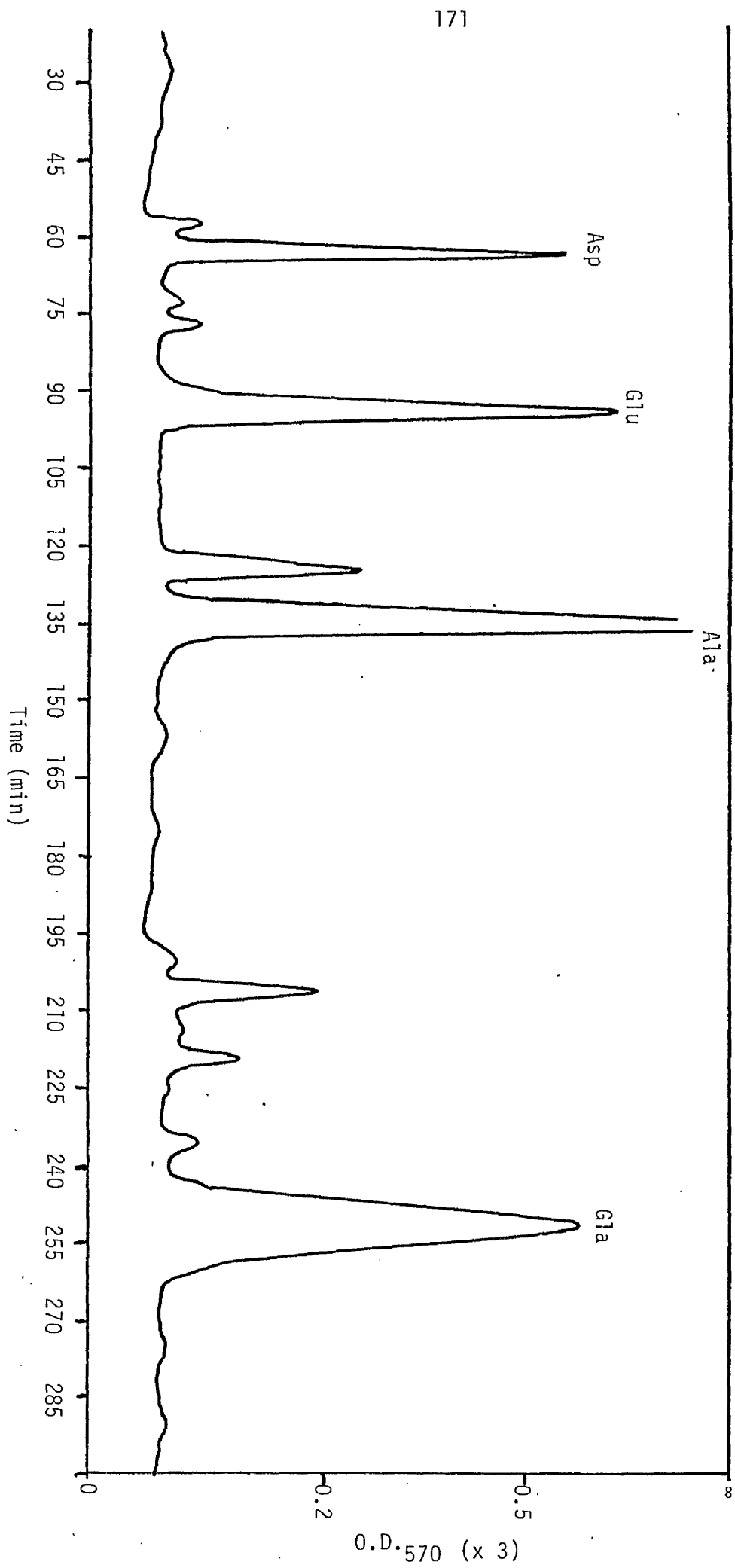


Figure 2.23      Amino sugars, amino acids and amino sugar derivatives in cell walls of *Str. lactis* C10 incubated with  $\phi$ C2(W)-induced lysin

Cell walls were hydrolysed using Method C in Methods. Concentration of cell walls used for analysis was 1.0 mg/ml.

Figure 2.23



The quantitative analytical data for the various cell wall constituents or their derivatives, with the exception of lysine, obtained using the various hydrolysis methods are shown in Table 2.14. These results show that the glucosamine levels in enzyme treated and control cell walls are similar. It is also apparent that the muramic acid content of enzyme-treated walls is considerably reduced compared with control walls. The different hydrolysis conditions also affected the concentration of glucosamine and amino acids detected. In particular higher levels of glucosamine were obtained if limited time and low acidity hydrolysis conditions were used. The cell walls of *Str. lactis* C10 contain the amino acids alanine, glutamic acid and alanine and most probably an unidentified basic amino acid which would not have been found under the analytical conditions used in these experiments.

Table 2.14

Quantitative analysis<sup>(1)</sup> of cell walls of *Str. lactis* C10 incubated with or without phage lysin and hydrolysed under differing conditions

Constituent	Hydrolysis conditions:			
	24 h, 110°C, 6 M HCl	4 h, 110°C, 6 M HCl	4 h, 95°C, 2.4 M HCl	
	µM/mg cell walls			
	- Enzyme	+ Enzyme	- Enzyme	+ Enzyme
Muramic acid <sup>(2)</sup>	0.10	0.015	0.21	0.046
Glucosamine	0.16	0.15	0.44	0.48
Muramicitol	0	T		(3)
Alanine	0.43	0.39	0.44	0.44
Glutamic acid	0.31	0.28	0.35	0.37
Aspartic acid	0.17	0.14	0.12	0.14
Glucosaminitol	0	0	0	0

T Not conclusively identified; concentration too low to quantify.

(1) Analyses performed using J. L. amino acid analyser.

(2) No allowance made for degradation during hydrolysis; results quantified using unhydrolysed muramic acid as standard.

(3) Unidentified substance present in the position where the muramicitol peak occurs - see Figure 2.22.

## DISCUSSION

The lytic agent present in  $\phi$ C2(W) lysates was isolated by ion exchange chromatography and was further purified by processes involving one or more of the following, ammonium sulphate precipitation, gel filtration and ultrafiltration. The characterisation studies established that the lytic agent was a basic substance which had similar properties to phage-induced lytic enzymes previously described for lactic streptococci (Reiter and Oram, 1963; Oram and Reiter, 1965; Tourville and Johnstone, 1966; Tourville and Tokuda, 1967) and other bacterial groups (Tsugita, 1971).

Lytic activity could not be resolved into more than one component on the basis of basicity or molecular geometry. This finding and the high recovery of lytic activity during purification suggests but does not prove that only one lytic enzyme is present in  $\phi$ C2(W) lysates.

Although appreciable purification and phage removal were obtained by the isolation methods, purified lysin preparations contained high levels of phage. This has also been found by others (Oram and Reiter, 1965; Tourville and Tokuda, 1967). If a method can be found to elute lysin from the dye ligand Green A, then the production of phage-free, lysin preparations may be attainable. Phage levels in purified enzyme preparations could probably be dramatically reduced by ultracentrifugation (Tourville and Tokuda, 1967). Since many phage lysins do not react with antiserum produced against the phage (Tsugita, 1971) the addition of phage antiserum to lysin preparations and the removal of the phage-antibody complex by centrifugation may also have value as a method for reducing the concentration of phage in lysin preparations. In this context it may be convenient to couple the antibody to a suitable matrix material e.g. glass beads and to use negative chromatography.

Purified lactic streptococcal phage lysins have been reported to be unstable (Oram and Reiter, 1965; Tourville and Tokuda, 1967) and the data obtained for  $\phi C2(W)$  lysin supports these findings. Nevertheless good stability of crude enzyme preparations obtained by ion exchange chromatography was found and little loss of activity was found in highly purified preparations held at  $-30^{\circ}\text{C}$  over a  $2\frac{1}{2}$ -year period.

In common with many other phage lysins,  $\phi C2(W)$  lysin was activated by cations. The activation of lactic streptococcal phage lysins by  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  has been reported previously (Oram and Reiter, 1965; Tourville and Tokuda, 1967) but this is the first report of activation by  $\text{Co}^{++}$  and  $\text{Mn}^{++}$ . The activation of lactic streptococcal phage lysins by divalent cations is in contrast to the marked inhibition of a group C streptococcal phage lysin by divalent cations ( $\text{Mg}^{++}$ ,  $\text{Ca}^{++}$ ,  $\text{Fe}^{++}$ ,  $\text{Mn}^{++}$ , or  $\text{Cu}^{++}$ ) reported by Barkulis *et al.* (1964). The  $\phi C2(W)$  lysin, however, differed qualitatively and quantitatively from other Group N streptococcal phage lysins in its response to cations. Lower concentrations of  $\text{Na}^{+}$ ,  $\text{K}^{+}$ ,  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  were required for maximum lytic activity (0.1, 0.1, 0.005 and 0.005 M respectively) compared with  $\phi ML3$  lysin. Oram (1964) and Oram and Reiter (1965) reported that  $\phi ML3$  lysin demonstrated maximum activity with 0.15 M concentrations of  $\text{Na}^{+}$  and  $\text{K}^{+}$  and 0.01 M concentrations of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ . Considerably higher concentrations of monovalent than divalent cations were required for maximum lytic activity for  $\phi C2(W)$  lysin and  $\phi ML3$  lysin (Oram and Reiter, 1965). Similar findings have been reported by Jensen and Kleppe (1972) and by Welker (1967) for T4 phage lysozyme and TP-1 lytic enzyme respectively. In contrast Tourville and Tokuda (1967) found that  $\phi C10$  lysin required higher concentrations of  $\text{Ca}^{++}$  than either  $\text{Na}^{+}$  or  $\text{Li}^{+}$  for maximum activity.

Oram and Reiter (1965) stated that high concentrations of divalent cations were less inhibitory than monovalent cations. High concentrations (0.4 M) of calcium chloride or magnesium chloride were reported not to inhibit the  $\phi$ C2 and  $\phi$ C10 lysins studied by Tourville and Tokuda (1967). In contrast the  $\phi$ C2(W) lysin was markedly inhibited by  $\text{Co}^{++}$ ,  $\text{Ca}^{++}$  and  $\text{Mn}^{++}$  concentrations slightly in excess of the levels required for maximum activation. Magnesium ions, however, were markedly less inhibitory. The response of  $\phi$ C2(W) lysin to activation by  $\text{Mg}^{++}$  and  $\text{Mn}^{++}$  is in accordance with the observation of Dixon and Webb (1964) who reported 'that in enzymes which are activated by both  $\text{Mg}^{++}$  and  $\text{Mn}^{++}$ , activation and inhibition are both shown at much lower concentrations of  $\text{Mn}^{++}$  than  $\text{Mg}^{++}$ '. Since the effect of ions on enzyme activation may be dependant on the age and purity of the preparation used (Dixon and Webb, 1964) caution should be exercised in comparing the effects of cations on the activity of partially purified enzymes.

The mechanisms involved in the activation of lactic streptococcal phage lysins by cations have not been studied. Possible mechanisms involved in enzyme activation by cations generally have been reviewed (Dixon and Webb, 1964). Since phage lysins are highly basic proteins (net positive charge) it is unlikely that cations act as binding links between enzyme and substrate combining with both and so holding the substrate at the active centre of the enzyme. Activation by  $\text{Na}^+$  and  $\text{K}^+$  would be difficult to explain if this mechanism were involved. Bacterial cell walls are elastic structures which shrink and swell in response to changes in the ionic strength or pH of the environment (Ou and Marquis, 1970). One explanation for the effect of cations on activation is that they induce the peptidoglycan to undergo conformational changes which expose susceptible linkages in the correct orientation for hydrolytic cleavage. For this hypothesis to be evaluated a substrate



which does not exhibit electromechanical properties is required. The isolation of disaccharides composed of muramic acid and glucosamine might yield a suitable substrate for such studies.

The  $\phi$ C2(W) lysin had a similar pH for optimum lytic activity to  $\phi$ ML3 lysin (Oram and Reiter, 1965) but differed significantly from the  $\phi$ C2 and  $\phi$ C10 lysins, studied by Tourville and Tokuda (1967), which demonstrated maximum lytic activity at pH 6-6.5.

The inactivation of lactic streptococcal phage lysins by mild heat treatment has been described previously (Naylor and Czulak, 1956; Oram and Reiter, 1965; Tourville and Tokuda, 1967). The results of this study provide further evidence for the difference in the thermal stability of lactic streptococcal phage lysins and phage. The heat lability of  $\phi$ C2(W) lysin and other lactic streptococcal phage lysins is in marked contrast to the heat stability of some lysozyme type enzymes (Jolles *et al.*, 1966; Eitenmiller, Friend and Shahani, 1975). Bovine milk lysozyme, for example, lost only 43% of its activity after 20 min at 100°C (pH 4.0). The thermal lability of lactic streptococcal phage lysins may explain why early studies of the nascent phage phenomenon did not find evidence for a lytic agent other than particulate phage (Collins, 1952a; Whitehead *et al.*, 1952; Whitehead *et al.*, 1953). Collins (1952a) investigated a phage which produced a nascent type reaction and attempted to determine whether the inhibition observed with phage lysates was due to phage *per se* or to another inhibitor. Since both phage and the ability of phage lysates to inhibit non-homologous hosts were lost after steaming for 3-4 min it was concluded that phage was directly involved in the inhibitory process. Whitehead *et al.* (1952) came to a similar conclusion which was partially based on the observation that both phage and the inhibitory agent present in a phage lysate were

destroyed by a heat treatment of 66°C for 5 min at pH 5. It is highly probable that both these heat treatments would have destroyed phage lysins. Hence it is possible that the nascent phage phenomenon observed by early investigators was due to phage lysins.

The  $Q_{10}$  and  $E_a$  values found in the present study for  $\phi C2(W)$ -induced lysin were significantly lower than values reported for other lactic streptococcal phage lysins (Oram and Reiter, 1965; Tourville and Tokuda, 1967). Both groups reported different values for phages active against *Str. lactis* C2. Since  $E_a$  and  $Q_{10}$  values are characteristic of the enzyme and not the substrate (Dixon and Webb, 1964) it is highly likely that  $\phi C2(W)$ ,  $\phi C2$  (Tourville and Tokuda, 1967) and  $\phi ML3$  (Oram and Reiter, 1965) lysins are different and also suggests that  $E_a$  and  $Q_{10}$  values may have a role in the differentiation of lysin-producing lactic streptococcal bacteriophages.

The results of this and other studies (Oram and Reiter, 1965; Tourville and Tokuda, 1967) suggest that lactic streptococcal phage lysins in common with many other phage lysins (see references of Tsugita, 1971) have a sulphydryl group or groups which is/are essential for lytic activity.

Unlike a Group C streptococcal phage lysin (Maxted, 1957; Doughty and Hayashi, 1962), the lytic activity of  $\phi C2(W)$  lysin was not significantly affected by reducing agents. In this context, the data from this study are in agreement with the results of Oram and Reiter (1965) and Tourville and Tokuda (1967). The response of Group N streptococcal phage lysins to reducing agents also appears to differ from a Group D streptococcal phage lysin which was inactive in the absence of reducing agents and required 0.7 M cysteine for optimal activity (Bleiweis and Zimmerman, 1961).

The sensitivity of hen egg lysozyme to inhibition by n-acetyl-muramic acid and n-acetylglucosamine has been suggested as a possible contributory factor in the insensitivity of certain bacteria to lysis by this enzyme (Rogosa, 1970). The insensitivity of  $\phi$ C2(W)-induced lysin to inhibition by both these amino sugars may contribute, at least in part, to its rapid lytic activity towards sensitive lactic streptococci. The reaction of  $\phi$ C2(W) lysin differed from that of T4 lysozyme to rivanol. Tsugita *et al.* (1968) successfully used this compound to precipitate DNA and acidic proteins during the purification of T4 lysozyme. Unlike the *E. coli* phage enzyme the streptococcal phage lysin was inhibited by rivanol.

The molecular weights of phage lysins produced by phages active against *E. coli* (Inouye and Tsugita, 1968; Moo-Penn and Wiesmeyer, 1969; Tsugita, 1971; Tsugita and Inouye, 1968), *Pseudomonas aeruginosa* (Bartell, Orr and Lam, 1966), a group C streptococcus (Barkulis *et al.*, 1964) and *Str. lactis* (Oram, 1964; Tourville and Tokuda, 1967) have been reported. The phage lysins produced by phages for *E. coli* were of low molecular weight, <19,000. Whereas, the lysins produced by the streptococcal phages had molecular weights in excess of 200,000 daltons. The lysin produced by the pseudomonas phage was reported to have a molecular weight of 180,000 daltons.

If phage lysin was associated with phage components e.g. tail fragments or tail fibres as found in a T2 lysin preparation by Katz (1964), molecular weight determinations could give erroneously high estimates of molecular weight. In this context some studies by Oram (1964) with  $\phi$ ML3 lysin are particularly interesting. Oram found that the phage lysin was inhibited by antiserum prepared against purified particles of the producing phage. This data suggests that  $\phi$ ML3 lysin

may be associated with phage components. If this is so, it is possible that the pure lysin may have a lower molecular weight than the value reported by Oram (1964). The  $\phi$ C2(W) lysin appeared to be free from contamination by phage components when studied by electron microscopy. If this is so, then the molecular weight estimate obtained in this investigation is probably within the error limits of the gel filtration technique. Andrews (1964) has reported that determinations by this method give molecular weight estimates with  $\pm 10\%$  of the actual value.

The  $\phi$ C2(W) lysin was found to lyse only streptococci belonging to Groups N and D. Strains of leuconstoc, *Str. thermophilus*, *Str. dysgalactiae* (group C), *Lact. bulgaricus*, *Lact. fermenti* and *E. coli* were not lysed. The classical lysozyme substrate *Micrococcus lysodeikticus* was not lysed either. The lytic activity of lactic streptococcal phage lysins towards group D streptococci has been reported previously (Oram and Reiter, 1965) and is confirmed by this work. This is the first report (to the best of my knowledge) concerning the failure of Group N streptococcal phage lysin to lyse strains of lactobacilli, *Str. thermophilus* and leuconstoc.

All strains of Group N streptococci studied were lysed. The wide lytic range of phage lysin compared with the relatively narrow lytic spectrum of particulate phage provides additional evidence for the role of lysins in the nascent phage phenomenon generally (see also Tourville and Tokuda, 1967) and in the involvement of lysin in the inhibition of acid production found when certain paired-strain cultures were infected with  $\phi$ C2(W) (p42). Strain-dependant differences in the lysis of Group N streptococci were found and confirm the findings of Oram and Reiter (1965) and Tourville and Tokuda (1967).

The lysin sensitivity of the components of the paired-strain cultures used to demonstrate the nascent effects of  $\phi C2(W)$  (p42) correlates well with the inhibition of acid production found in  $\phi C2(W)$ -infected cultures. Cultures which demonstrated little acid production when infected with  $\phi C2(W)$  e.g. C2+C10 contained component strains which were highly sensitive to lysin. Conversely the two cultures which showed little inhibition of acid production i.e. AM2+C2 and US3+C2 contained component strains which were relatively insensitive to phage lysin. The commercial significance of these findings have been discussed previously (p90).

Unlike  $\phi C10$  lysin (Tourville and Tokuda, 1967),  $\phi C2(W)$  and  $\phi ML3$  (Oram and Reiter, 1965) lysins failed to lyse *Micrococcus lysodeikticus*. The lytic reaction of streptococcal phage lysins towards this organism may therefore be useful in their differentiation and, indirectly, in the differentiation of lysin-producing phages.

The chemical and/or stereochemical factors which determine whether a particular phage lysin lyses one bacterial species while not lysing another are not well understood.

Differences in peptidoglycan structure may be important. Such differences would be expected to influence whether or not the lysin sensitive bond was accessible and/or in the correct position for cleavage. If this is so, then structural similarities would be expected between the peptidoglycans of Group N streptococci and those of the three species of Group D streptococci lysed by the phage lysin. Conversely differences in structure would be expected between the peptidoglycan of Group N streptococci and species which were not lysed e.g. *Str. thermophilus*.

The three dimensional structure of the peptidoglycan of members of the genus *Streptococcus* has not been determined. However, detailed information is available concerning the chemical structure of peptidoglycan types present in this genus (Schleifer and Kandler, 1972). Two types of peptidoglycan have been reported in Group N streptococci. The first type contains the peptide L-alanine-D-glutamine-L-lysine-D-alanine linked to muramic acid. In this type, the peptide is cross linked by D-isoasparagine between L-lysine at C<sub>3</sub> and D-alanine at C<sub>4</sub> on adjacent chains. In the second type, the crossbridge differs in that it is formed by the dipeptide L-alanyl-L-threonine (Schleifer and Kandler, 1967). However, the strains used to derive the latter type were probably incorrectly classified (Schleifer and Kandler, 1972) and it is highly probable that the first type represents the peptidoglycan present in the Group N streptococci. Of the species studied only *Str. faecium* and *Str. durans* possessed this type of peptidoglycan (Schleifer and Kandler, 1972). Since strains of these species were lysed, a relationship between the chemical structure of a peptidoglycan and its susceptibility to lysis by  $\phi$ C2(W) lysin may exist. However, *Str. faecalis* which was also lysed, has a peptidoglycan of identical chemical structure to *Str. thermophilus* which has not lysed. The peptidoglycan of both species is similar to that in the species discussed previously except for the possession of a different interpeptide crosslinkage - the tripeptide of L-alanine. In fact the chemical structure of the peptidoglycan of the members of the genus *Streptococcus* studied by the author for sensitivity to lysin is similar to that of the Group N streptococci apart from differences in interpeptide crosslinks (Schleifer and Kandler, 1972). Consequently insufficient information is available to relate the susceptibility of a species to lysis by phage lysin and the chemical structure of its peptidoglycan.

Although the chemical and ultimately the three dimensional structure of peptidoglycan must influence the sensitivity of a strain or a particular species to phage lysin, the nature of the non-peptidoglycan components may also be important. The nature of the non-peptidoglycan components influences the sensitivity of cell walls to hen egg lysozyme and may also influence sensitivity to phage lysins. Resistance to lysozyme has been attributed to the attachment to the peptidoglycan of polymers such as teichoic acid (Mandelstam and Strominger, 1961) or polysaccharide (Krause and McCarty, 1961). Much simpler constituents attached to amino sugar constituents for example, can also influence the sensitivity of cell walls to lysozyme and to phage lysins. Acetyl groups ester linked (O-acetyl groups) to the peptidoglycan in some strains of *M. lysodeikticus* have been shown to prevent the action of hen egg lysozyme (Brumfitt *et al.*, 1958; Logardt and Neujahr, 1975) and phage N1 lysin (Brumfitt, 1960). Ghuysen *et al.* (1966) have suggested that some lytic enzymes have strict substrate requirements with respect to the size of the polymer, the extent of crosslinking, the net electrical charge in addition to other unspecified requirements. It is not therefore unreasonable that  $\phi$ C2(W) and other phage-induced lysins have similar substrate requirements.

In a preliminary communication Reiter and Oram (1963) reported that phage and lysin competed for adsorption sites that were either identical or very closely linked. The basis for this statement was the observation that the addition of increasing multiplicities of phage to  $\phi$ ML3 lysin preparations resulted in a progressive inhibition of lysis by the lytic enzyme. If this is so then it can be argued that lysin should not attack a strain which does not possess receptor sites for the lysin-producing phage. Also, a relationship between lysin sensitivity and the number of phage receptor sites on a cell might also be expected. Tourville and Tokuda (1967) attempted to explain the

differences in sensitivity of various lactic streptococci to two phage lysins on the basis that both phage and lysin attacked the same receptor site and inferred that strain sensitivity differences may be related to the possession or absence of phage and hence lysin receptor sites on non-homologous strains.

The results of this study show that the phage lysin lysed strains which did not absorb or poorly absorbed  $\phi C2(W)$  and showed either high or low lytic activity towards strains which adsorbed the phage at high efficiency. No evidence was therefore found that phage and lysin competed for similar reaction sites. Furthermore the addition of high multiplicities of  $\phi C2(W)$  to phage lysin did not inhibit lytic activity. These results agree with work by Oram and Reiter (1965) who reported that the inhibition of lytic activity found in their earlier communication (Reiter and Oram, 1963) was not due to phage but to an unknown lysis inhibitor that was present in crude phage lysates. Definitive evidence for a lysin inhibitor present in lysates of  $\phi C2(W)$  was not found in the present study. It is possible that the increase in lytic activity frequently found during lysin purification was due to the removal of a lytic inhibitor. The basic substance isolated from  $\phi C2(W)$  lysates which was originally suspected of being a lysis inhibitor is probably nisin (Hurst, 1973; Hurst, 1978). Nisin is a highly basic polypeptide which is produced by *Str. lactis* strains. It is virtually insoluble at pH values close to neutrality and demonstrates antimicrobial activity against Gram-positive bacteria.

Ultrastructural studies were used to confirm that the cell wall was the site of action of the phage enzyme. The entire cell surface appeared to be readily accessible to lysin action and no evidence was found to suggest that the enzyme attacked only specific areas as would be expected if lysin just attacked phage receptor sites. The electron



micrographs obtained were similar to some published by Siegel *et al.* (1981) who studied the action of mutanolysin on *Str. mutans*. However, the phage lysin was dramatically more effective at removing the cell walls of *Str. lactis* C10 than mutanolysin was for *Str. mutans*. The  $\phi$ C2(W) enzyme gave a similar degree of wall removal in 100s, to that obtained with mutanolysin in 15-30 min (see Figure 2 of Siegel *et al.*, 1981). The effectiveness with which phage lysin removed the cell walls of sensitive streptococci helps to explain further the inhibition of acid production in milk-based media and the inhibition of growth or cell lysis found in broth when certain paired or multi-strain cultures were infected with  $\phi$ C2(W) - see Chapter 1; the host strain produced lysin, which removed the cell wall of phage-resistant component strains, creating protoplasts. Acid production and growth were inhibited, and cell lysis which was observed by a reduction in absorbance in broth media (p. 11) occurred because the protoplasts lysed and died in the non-isotonic environment.

Further evidence that the cell wall was the site of action of the  $\phi$ C2(W) lysin was obtained from the lysis of purified cell walls by the phage lysin.

The phage lysin was shown to be similar to both  $\phi$ ML3 lysin (Oram and Reiter, 1965) and the group C streptococcal lysin studied by Barkulis *et al.* (1964) in that it attacked amino sugar linkages in the peptidoglycan. Unlike the group C lysin,  $\phi$ C2(W) lysin was not a N-acetylglycosidase and by inference is an N-acetylmuramidase. Proof that an enzyme is a muramidase would be provided if its action results in a reduction of the muramic acid content of suitably treated cell walls and the production of muramicitol. An apparent reduction in the muramic acid concentration was found but the presence of muramicitol in reduced enzymic digests

was not unequivocally proven. Nevertheless the demonstration that the enzyme was a glycosidase, that the glucosamine linkage was not cleaved, combined with the suggestion that muramicitol was present in reduced enzyme treated wall digests are consistent with the lysin being an n-acetylmuramidase.

The failure to demonstrate unequivocally that the lysin was a muramidase was due to the inability of the analytical technique used to adequately resolve muramic acid and glutamic acid, and aspartic acid and muramicitol, the poor colour response of the muramicitol-ninhydrin compound and the lability of muramic acid during hydrolysis. That the muramicitol-ninhydrin complex gives a very low colour response in the amino acid analyser has been reported (Tsugita *et al.*, 1968). Although the previous workers used a similar analytical technique they did not report difficulties in the separation of the amino sugars and their derivatives from aspartic acid and glutamic acid or problems in the determination of muramic acid. In view of our experience (Mullan and Manson, unpublished information) the results of Tsugita *et al.* (1968) are difficult to explain and further work is suggested.

Cell walls are also degraded by autolytic enzymes. Autolytic enzymes of streptococci of Groups N and D have been isolated and characterised recently. It is of interest to compare the properties of the streptococcal autolysins with the  $\phi$ C2(W)-induced lysin. The autolysin of *Str. faecalis* is like  $\phi$ ML3 lysin and probably  $\phi$ C2(W) lysin, a muramidase (Shockman, Thompson and Conover, 1967). However, partially purified autolysin, unlike the lactic streptococcal phage lysins, does not require reduced sulphydryl groups for activity. A further difference between  $\phi$ C2(W) lysin and the *Str. faecalis* autolysin is that the latter is inhibited by nanomolar concentrations of cardiolipin

(Cleveland *et al.*, 1975; Cleveland *et al.*, 1976; Shungu *et al.*, 1980) whereas the former is not affected. The autolysins of some *Str. cremoris* strains have been studied by Mou, Sullivan and Jago (1976). A pH optimum between 6.5 and 7.0 was reported for one strain and a value between 6.0 and 6.5 was reported for a second strain. The effect of temperature on the autolytic activity of two strains was studied. Both strains demonstrated a high rate of autolytic activity at 45°C. This is in marked contrast to the response of  $\phi$ C2(W) lysin or to  $\phi$ ML3 and  $\phi$ C10 lysins (Oram and Reiter, 1965) at these temperatures; all three enzymes are inactivated at this temperature. Unlike the phage lysins, the autolysins of two *Str. cremoris* strains were not inhibited by high concentrations of  $\text{Na}^+$ .

Despite the fact that extensive characterisation of Group N streptococcal autolysins has not yet been undertaken the preliminary study cited above suggests that there are qualitative and quantitative differences between the autolysins and the phage-induced lysins studied to date and suggests that phage lysins are not autolytic enzymes that have increased in concentration due to phage infection. Data is now available concerning the activity of lysins produced by three phages active against *Str. lactis* C2 i.e.  $\phi$ C2(W) lysin,  $\phi$ C2 lysin (Tourville and Tokuda, 1967) and  $\phi$ ML3 lysin (Oram and Reiter, 1965).  $\phi$ ML3 lysin is included because *Str. lactis* strains C2 and ML3 are thought to be virtually identical (Davies *et al.*, 1981) a fact that was unknown at the start of this project. All three lysins differ qualitatively and quantitatively from one another, a fact which supports the argument that lactic streptococcal phage lysins differ from autolytic enzymes present in lactic streptococci; if this were otherwise a similar enzyme would be expected in lysates of all three phages.

### SUMMARY

The lytic agent present in  $\phi$ C2(W) lysates was isolated by ion exchange chromatography using Amberlite CG50 and further purified using ultrafiltration or gel filtration.

The lytic enzyme was active over a broad pH range with an apparent pH optimum of 6.5-6.9. Purified lysin was unstable in 0.1 M potassium phosphate buffer at temperatures  $>30^{\circ}\text{C}$  and only 3.4% of the activity remained after incubation for 5 min at  $47^{\circ}\text{C}$ . An apparent temperature optimum of  $37^{\circ}\text{C}$  was found and  $Q_{10}$  and  $E_a$  values over the range  $22-32^{\circ}\text{C}$  were 2.5 and  $69.2 \text{ kJ mole}^{-1}$  respectively. The enzyme was activated by both monovalent and divalent cations; higher concentrations of monovalent cations were required for activation. No specific anion requirement was found.

Reduced sulphydryl groups were shown to be required for lytic activity. Concentrations of N-acetylglucosamine up to 50 mM, did not significantly affect lytic activity. Gel filtration using a calibrated column of Bio Gel P100 revealed a molecular weight of about 46,000.

All strains of Group N streptococci studied were lysed, however, strain dependent differences in lysin sensitivity were apparent. Group D streptococci were also lysed. Strains of *Leucon. cremoris*, *Leucon. lactis*, *Leucon. dextranicum*, *Str. thermophilus*, *Lact. bulgaricus*, *Lact. fermenti*, *E. coli*, *Micrococcus lysodeikticus* and *Str. dysgalactiae* were apparently resistant. No relationship was found between the ability of a strain to absorb  $\phi$ C2(W) and its sensitivity to lysis by  $\phi$ C2(W)-induced lysin; strains which did not absorb the phage showed high sensitivity to lysin. Examination of lysed cells by electron microscopy revealed that lysin removed the cell walls of sensitive streptococci creating protoplasts.

Examination of cell wall degradation products revealed that the enzyme was a glycosidase. Analysis of reduced enzyme treated cell walls, using an amino acid analyser, revealed that the concentration of muramic acid was reduced compared with control walls. Muramicitol was not definitely detected. However, the glucosamine linkage was not attacked and it is probable that the phage enzyme is a N-acetylmuramidase.

The  $\phi C2(W)$  enzyme is compared with autolytic enzymes described for streptococci of Groups N and D because of qualitative and quantitative differences between the phage-induced enzyme and autolytic enzymes it is unlikely that the  $\phi C2(W)$  enzyme is an autolytic enzyme which has increased in concentration as a result of phage infection.

### CHAPTER 3

EFFECT OF CULTURAL AND OTHER FACTORS ON THE SENSITIVITY  
OF *Str. lactis* C2 AND SOME OTHER STREPTOCOCCI TO PHAGE LYSIN

## INTRODUCTION

With the exception of the report that the ageing of *Str. cremoris* KH cells led to a progressive resistance towards lysis by  $\phi$ ML3 lysin (Oram and Reiter, 1965), the factors affecting the sensitivity of Group N streptococci to phage lysins have not been reported. Limited data is available, however, concerning the factors which affect the sensitivity of Group N streptococci and other bacterial groups to hen egg lysozyme (E.C.3.2.1.17).

Because  $\phi$ C2(W) lysin, like hen egg lysozyme, appears to be a N-acetylmuramidase factors influencing the sensitivity of lactic acid bacteria to lysozyme will be briefly reviewed since they may also apply to the phage enzyme.

Addition of the amino acids L-threonine (or DL-threonine) and L-lysine to growth media has been reported to give cells of increased sensitivity of lysozyme (Chassy, 1976; Chassy and Giuffrida, 1980).

The growth phase of cells has also been found to influence sensitivity. Both Neujahr (1966) and Shockman, Thompson and Conover (1967) reported that washed cells or cell walls of *Lact. fermenti* were not sensitive to lysozyme. However, further work by the former worker established that the almost complete resistance of this organism to lysozyme was only valid for stationary phase cells whereas cells taken from the log phase were much more sensitive (Neujahr, Borstad and Logardt, 1973). Log phase cells of *M. lysodiecticus* and *Lact. plantarum* have also been shown to be more sensitive to lysozyme than stationary phase cultures (Brumfitt, Wardlaw and Park, 1958; Holden and van Balgooy, 1965). Log phase or very early stationary phase cells of *Str. lactis* have been used preferentially in protoplast fusion and plasmid studies (Davies and Gasson,

1981) presumably due to their increased sensitivity to lysozyme. However, Chassy and Giuffrida (1980) found that stationary phase cells of *Lact. casei* and *Str. mutans* were more sensitive to lysozyme than log phase cells.

The substrate on which cells are grown may also influence lysozyme sensitivity. Chassy and Giuffrida (1980) grew cells in media containing D-glucosamine, D-gluconate, L-malate, pyruvate, ribitol, and citrate and found glucose-grown cells most sensitive to lysozyme.

Most cell suspensions undergo a slight decrease in turbidity with incubation time due to autolysis. Shockman *et al.* (1968) have attributed the increased lysozyme sensitivity of log phase cells of *Str. faecalis* compared with stationary phase cells, in part, to the greater autolytic activity of the log phase cells.

That research into the genetics of Group N streptococci and other lactic acid bacteria has made significant progress is apparent from the review by Davies and Gasson (1981). The existence of plasmid DNA in both lactic streptococci and lactobacilli is now well established. Several important properties of cheese starters, e.g. lactose, proteinase, citrate and nisin metabolism are thought to be plasmid associated. Gene transfer by the induced fusion of bacterial protoplasts and subsequent regeneration of complete cells on hypertonic media has been recently described for various strains of Group N streptococci, *Str. thermophilus* and *Str. faecalis* (Gasson, 1980). The demonstration of plasmid DNA and the production of protoplasts both require a gentle method of cell wall removal. Although procedures for cellular lysis of lactic streptococci with lysozyme have been described (Klaenhammer, McKay and Baldwin, 1978; Gasson, 1980) these methods have not been effective for all strains, especially those



of *Str. cremoris*. In addition the extended incubation time required to obtain cell lysis with lysozyme may result in the destruction of plasmid DNA by host nuclease activity, e.g. loss of the lactose plasmids in *Str. lactis* C2 and *Str. cremoris* B1 occurs after 20 min incubation at 37°C with lysozyme (Klaenhammer *et al.*, 1978).

Characterisation studies (Chapter 2) undertaken with  $\phi$ C2(W) lysin established that the enzyme rapidly lysed a range of lactic streptococci and had a similar mode of action to lysozyme. This suggested that this enzyme might be a more effective cell wall removing agent, at least for Group N streptococci, than lysozyme. If  $\phi$ C2(W) lysin is to be used for this purpose it would be desirable to have information concerning factors which influence the sensitivity of lactic streptococci to the lytic enzyme. In this section the effect of growth phase, carbohydrate substrate, temperature of growth, agents which affect peptide cross linking, freeze drying, autolytic rate, and concentration of osmotic stabiliser on the sensitivity of *Str. lactis* C2 and other streptococci to  $\phi$ C2(W)-induced lysin is reported.

## MATERIALS AND METHODS

### CULTURES

Strains C2, C10, ML8, AM1, AM2, RM1 SK11, US3, 1249 and DRC2 were used. The origins of the cultures and the propagation conditions used have been described previously (p20 and p99).

### MEDIA AND GROWTH OF CULTURES

Strains were grown in PLGYG broth (Mullan *et al.*, 1981b), M17 (Terzaghi and Sandine, 1975), MRS broth (de Man, Rogosa and Sharpe, 1960) and lysis broth (Klaenhammer *et al.*, 1978). Cultures for experiments were obtained by inoculating cells previously grown in PLGYG into the appropriate medium which was incubated at 22°C or 30°C for a designated time. For cultures incubated at 22°C a 1% inoculum (v/v) was used. A lower inoculum (0.1%) was used for strains grown at 30°C unless stated otherwise. All cultures received two transfers in the appropriate medium prior to use in lysis experiments.

### FREEZE-DRIED CULTURES

Strains were grown in PLGYG broth (1% inoculum) at 22°C for 16 h and harvested by centrifugation. Cells were washed twice with distilled water and suspended in a small quantity of distilled water. The resultant slurry, generally less than 10 ml, was frozen at -18°C overnight (18 h) and freeze-dried as described previously (p25). Although cultures appeared to be dry at 24 h, drying was continued for a further 24 h. Freeze-dried cells were stored in a desiccator at 5°C until required.

## CHEMICALS

All chemicals used were of 'Analar' grade or highest purity available. L-lysine, L-threonine and galactose were obtained from Sigma. Sucrose, sodium gluconate, penicillin G, glucose and maltose were obtained from BDH.

## PHAGE LYSIN

Partially purified  $\phi$ C2(W)-induced lysin was used. The enzyme was purified by ion exchange, ammonium sulphate precipitation, gel filtration and was desalted by ultrafiltration. The preparation used had a protein content of 35  $\mu$ g/ml and gave an activity of 25,000 u/ml using freeze-dried cells of C2 as described previously (p24).

## SENSITIVITY OF CELLS TO LYSIN

The procedure used was similar to that used for the assay of lysin activity described previously except that the results were expressed in a slightly different format. Cells were harvested from growth media, washed once in 0.1 M phosphate buffer and suspended in 0.1 M phosphate buffer to give an O.D. 450 of 0.65-0.70 in the Bausch and Lomb Clinical Analyser or the Shimadzu Spectrophotometer. A standardised procedure was adopted in all experiments. To 3.0 ml of cell suspension 25  $\mu$ l of lysin were added. The mixture was briefly mixed and the decrease in O.D. 450 with time at 37°C recorded. The linear part of the O.D. 450 versus time plot was calculated and used to calculate the  $\Delta$  O.D./min. In previous experiments this figure would have been used to calculate enzyme activity as follows:

$$\frac{\Delta \text{ O.D./min}}{0.001 \times 0.025} = \text{lysin activity (u/ml)}$$

Therefore the  $\Delta$  O.D./min values can be multiplied by 40,000 to give lysin activity in u/ml if the reader wishes to compare the results in this section with those in the previous sections.

#### AUTOLYTIC ACTIVITY

Autolytic activity was measured in a similar way to lysin activity except that no lytic enzyme was used. In these experiments the O.D. 450 of cell suspensions was adjusted to about 0.7 and the change in absorbance recorded over a 4 min period. Provided that both spectrophotometers had been switched on for at least  $1\frac{1}{2}$  h 'Instrument drift' was less than 0.001 O.D. units/10 min.

## RESULTS

### EFFECT OF GROWTH PHASE

The effect of growth phase on the sensitivity of C2 and other streptococci to lysin is shown in Table 3.1. Logarithmic phase cells were lysed at a faster rate than stationary phase cells. That log phase cells of ML8 were particularly sensitive to lysin is apparent. During repeat experiments it was found that slight differences in incubation time had a significant effect on lysin sensitivity. This finding prompted a more detailed investigation of the effect of growth phase on lysin sensitivity (Table 3.2). The results confirmed that log phase cells of C2 were more susceptible to lysis than stationary phase cells but revealed that early log phase cells (4 h) were particularly sensitive. Although the data presented in Tables 3.1 and 3.2 show that stationary phase cells were less sensitive to lysin, they were lysed ultimately to the same extent as log phase cells.

The autolysis rate of the cells used in the assays is also presented in Table 3.2. Highest autolytic activity was found in the 8½ h cells (about early stationary phase). No correlation between lysin sensitivity and autolytic rate was found for C2.

### EFFECT OF CARBOHYDRATE SOURCE

The effect of substrate and incubation temperature on the sensitivity of C2 cells to lysin is shown in Table 3.3. Satisfactory growth was obtained with all energy sources but initial growth on gluconate was slower than with other substrates. It is apparent that the energy source used had a marked influence on lysin sensitivity. Media containing glucose yielded cells which were more sensitive to lysin than cells obtained from media containing other carbohydrates. Gluconate gave the least

Table 3.1

Effect of growth phase on the sensitivity of lactic  
streptococci to phage lysin

Strain	$\Delta$ O.D./min	
	Log phase	Stationary phase
C2	0.286	0.16
C10	0.554	0.447
ML8	0.60	0.275

Cells were grown in PLGYG broth for 6 h (1%, 30°C) and for 18 h (1%, 30°C), harvested and their susceptibility to lysis determined as in Methods.

Table 3.2

Effect of age of cells of *Str. lactis* C2 on lysin sensitivity and autolytic rate

Time (h)	$\Delta$ O.D./min	$\Delta$ O.D./4 min
	+ lysin	- lysin
4	0.53	0.001
5	0.37	0.003
6	0.25	0.002
7	0.26	0.003
8½	0.22	0.008
24	0.33	0.002

Cells were grown in PLGYG broth (1% inoculum) at 30°C, harvested at intervals and susceptibility to lysin and their rate of autolysis determined as in Methods.

Table 3.3

Effect of carbohydrate source and growth temperature on the sensitivity of cells of *Str. lactis* C2 to phage lysin

Substrate	$\Delta$ O.D./min					
	Glucose	Galactose	Maltose	Fructose	Lactose	Gluconate
22°C	0.36	0.085	0.18	0.16	0.20	0.02
30°C	0.39	0.115	0.12	0.23	0.33	0.022

Cells were grown in PLGYG broth in which the glucose component was replaced with the appropriate sugar and grown to late stationary phase at 22°C or 30°C. Cells were harvested and lysin sensitivity determined as in Methods.



sensitive cells. Cells grown in media containing galactose, maltose, fructose and lactose yielded cells which were less sensitive to lysis than glucose-grown cells but were more sensitive than cells grown on gluconate.

In general, incubation temperature (22°C, 30°C) had little effect on sensitivity to lysin. Cells grown at 30°C, particularly cells grown on lactose, were frequently slightly more sensitive.

The autolysis rate was less than 0.001 O.D. units/4 min for cells from all substrates. No correlation was found between autolysis rate and lysin sensitivity.

Because of the low sensitivity of cells grown on gluconate to lysin, further study of C2 cells grown on gluconate was undertaken. Log phase cells (1% inoculum 30°C, 6 h) were significantly more sensitive to lysis giving a  $\Delta$  O.D./min of 0.081 compared with a  $\Delta$  O.D./min of 0.02 for stationary phase cells (22°C, 1%, 16 h). This result confirmed that log phase cells were more sensitive to lysin than stationary phase cells. Attempts were made to determine whether the low sensitivity of cells grown on gluconate to lysin was due to the selection of a mutant population or due to a chemical change, perhaps in cell wall composition. In these experiments C2 cells were grown in broth containing gluconate for three transfers (1%, 22°C, 16 h) or in PLGYG and used to inoculate fresh media as shown in Table 3.4. The glucose-grown cells transferred to PLGYG exhibited high sensitivity to lysin whereas similar cells grown in gluconate for 6 h exhibited low sensitivity to lysin. The cells grown on gluconate for three transfers and transferred to fresh media containing gluconate gave similar lysin sensitivity results to the glucose-grown cells transferred to gluconate and tested after 6 h. These results suggest that growing

Table 3.4

Effect of transferring glucose or gluconate-grown cells of  
*Str. lactis* C2 to media containing glucose or  
 gluconate on lysin sensitivity

Treatment	$\Delta$ O.D./min
Glucose-grown cells transferred to glucose	0.3
Glucose-grown cells transferred to gluconate	0.081
Gluconate-grown cells transferred to glucose	0.144
Gluconate-grown cells transferred to gluconate	0.081

Cells were grown in PLGYG or a similar medium, in which gluconate replaced glucose, for 3 transfers prior to experiments. Cells were inoculated into the appropriate medium (1% inoculum) and their lysin sensitivity determined after 6 h at 30°C.

C2 cells on gluconate does not result in the selection of a mutant population which is more resistant to lysis than glucose-grown cells; significant growth of a mutant population in 6 h is improbable. If this hypothesis is correct, transfer of cells, previously grown on gluconate, to media containing glucose should result in cells of increased sensitivity to phage lysis. The results obtained revealed that such cells were more sensitive to lysin action than control cells grown on gluconate but were less sensitive than cells grown on glucose and transferred to fresh media containing glucose.

#### EFFECT OF AGENTS WHICH AFFECT PEPTIDE CROSS LINKS IN THE PEPTIDOGLYCAN

##### Effect of L-lysine and L-threonine

The sensitivity of cells grown in PLGYG broth supplemented with lysine, threonine and/or a mixture of both amino acids was studied (Table 3.5). Threonine supplementation did not increase the sensitivity of C2 to lysin. In most experiments, media containing threonine yielded cells which had a reduced sensitivity to lysis by the phage-induced enzyme. Threonine supplementation had no apparent affect on the sensitivity of DRC2 cells to  $\phi$ C2(W) lysin. Lysine supplementation consistently gave DRC2 cells which were more sensitive to lysis and yielded C2 cells which had a slightly increased sensitivity to the phage-induced enzyme. No positive synergistic effect on lysin sensitivity was found for cells grown in media containing both amino acids.

##### Effect of growing cells in sub-lethal concentrations of penicillin

The sensitivity of C2 cells grown in sub-lethal concentrations of penicillin to lysin is shown in Table 3.6. Because the results were

Table 3.5

Effect of growing cells in PLGYG supplemented with  
L-lysine and/or L-threonine on lysin sensitivity

Strain <sup>(2)</sup>	$\Delta$ O.D./min with addition to PLGYG <sup>(1)</sup>			
	None	+ lysine	+ threonine	lysin + threonine
C2	0.314	0.345	0.239	0.279
DRC2	0.005	0.01	0.006	0.013

(1) PLGYG was supplemented with 10 mM lysine, 10 mM threonine or 5 mM of each as indicated above.

(2) Cells were grown to log phase (1%, 30°C, 6 h), harvested and examined for sensitivity to lysin.

Table 3.6

Effect of growing cells of *Str. lactis* C2 in media containing penicillin G on lysin sensitivity and autolytic rate

Penicillin concentration (IU/ml)	0	0.1	0.15
Lysis with phage lysin $\Delta$ O.D./min	0.234	0.431	0.718
Autolytic rate $\Delta$ O.D./4 min	<0.001	<0.001	<0.001

Cells were inoculated into PLGYG broth (1% inoculum) with or without antibiotics and grown at 22°C for 16 h. Cells were harvested and their lysin sensitivity and autolytic rate determined as in Methods.

so dramatic the results are also expressed graphically in Figure 3.1. As far as C2 was concerned, no inhibition of acid production was found with penicillin concentrations of 0.1 and 0.15 IU/ml. A marked increase in sensitivity to lysis was found for cells grown in the presence of 0.1 IU/ml. A further almost two-fold increase in lytic rate was found for cells grown in 0.15 IU/ml of penicillin. The autolysis rate was measured for control and for cultures grown in penicillin. The rate was similar for all cultures tested and was less than 0.001 O.D. units over a 4 min period.

Further experiments were undertaken to determine whether growth in sub-lethal concentrations of penicillin could be used to increase the sensitivity of other lactic streptococci to  $\phi$ C2(W)-induced lysis. In these experiments flasks of PLGYG broth containing 0.15 IU penicillin/ml were inoculated with 1% AM1, AM2, DRC2 and C10 respectively and the cells were harvested after 6 h at 30°C. Little or no growth of the *Str. cremoris* strains occurred but C10 and DRC2 were apparently unaffected. The results (Table 3.7) showed that sub-lethal concentrations of penicillin also increased the sensitivity of DRC2 and C10 to lysis by the phage lysin.

#### EFFECT OF GROWTH MEDIUM

Strains were grown in PLGYG, M17 and MRS and in lysis broth for 6 h at 30°C (1% inoculum), harvested and their sensitivity to lysin determined (Table 3.8). PLGYG broth yielded cells of C2 which were more sensitive to lysis than the other media studied. MRS broth yielded the least sensitive cells to phage lysin. M17 broth yielded cells of AM1 and DRC2 which were more sensitive to  $\phi$ C2(W) lysin than the other media tested. As with C2, MRS yielded cells which were least sensitive to

Figure 3.1 Lysin sensitivity of cells of *Str. lactis* C2 grown in media containing different concentrations of penicillin G

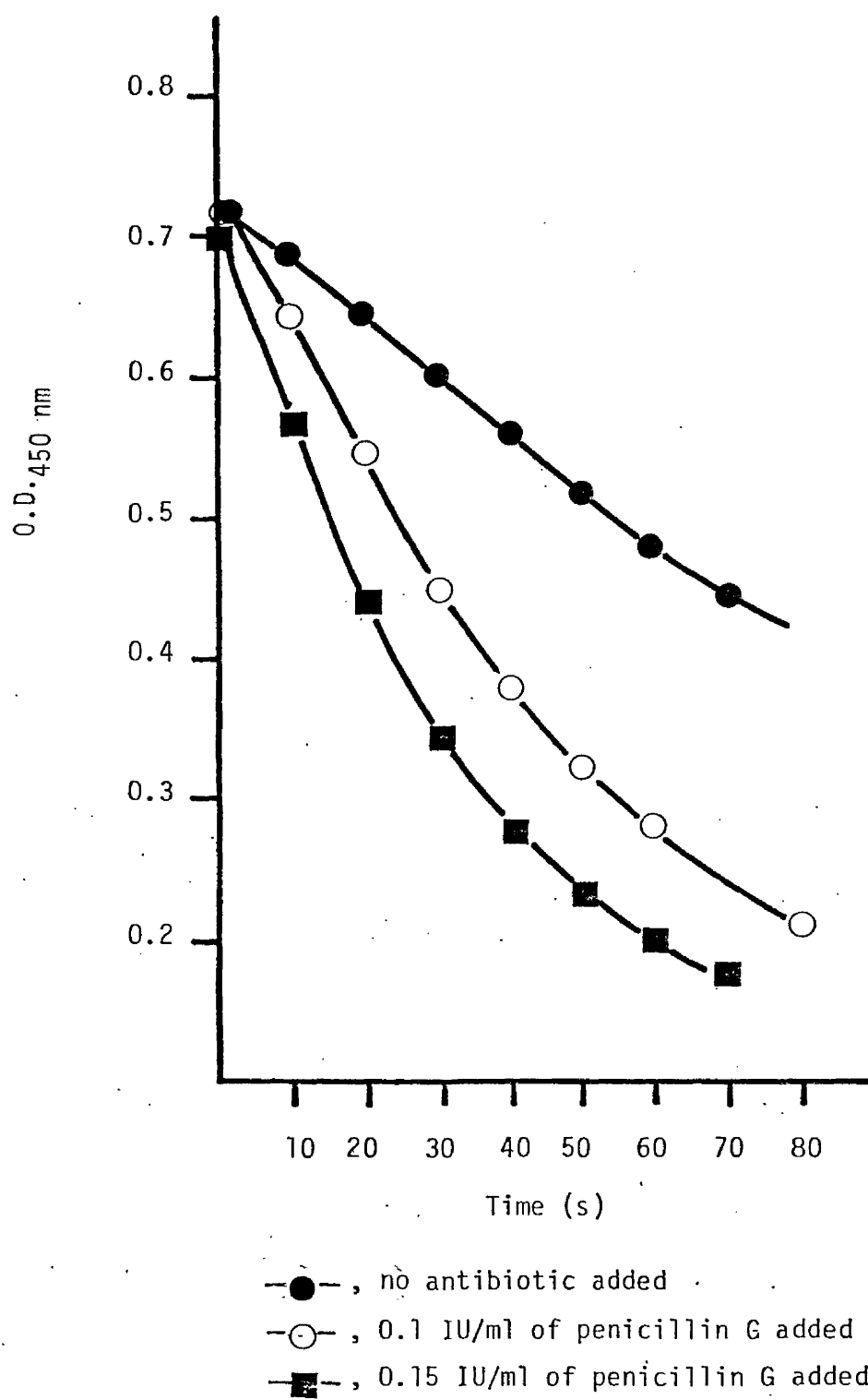


Table 3.7

Effect of growing cells of *Str. lactis* C10 and *Str. diacetylactis* DRC2  
in media containing penicillin G on lysin sensitivity

Penicillin concentration (IU/ml)	0	0.15
DRC2 ( $\Delta$ O.D./min)	0.005	0.04
C10 ( $\Delta$ O.D./min)	0.554	0.812

Experimental conditions were similar to those described in Table 3.6 except that log phase cultures (1% inoculum, 6 h, 30°C) were used.



Table 3.8

Effect of growth medium on the sensitivity of  
lactic streptococci to phage lysin

Strain <sup>(1)</sup>	$\Delta$ O.D./min			
	PLGYG	M17	MRS	Lysis broth
C2	0.410	0.166	0.016	0.201
AM1	0.053	0.181	0.011	0.067
DRC2	0.007	0.011	0.002	0.005

(1) Cells were grown in the medium indicated for 6 h at 30°C (1% inoculum), harvested and their sensitivity to lysin determined.

lysin. This experiment was repeated several times and similar qualitative results were obtained.

#### EFFECT OF FREEZE-DRYING

Preliminary experiments established that freeze-drying markedly increased the sensitivity of C2 to the phage enzyme. Attempts were made to determine if this method could be used to increase the sensitivity of other Group N streptococci to lysis by  $\phi$ C2(W) lysin. The results (Table 3.9) reveal that freeze-drying markedly increased the sensitivity of all strains tested to lysin. Further study revealed that the freezing process did not affect lysin sensitivity. Freshly harvested cells and cells frozen for various times had similar sensitivities to lysin.

#### EFFECT OF SUCROSE

Sucrose is frequently used as an osmotic stabiliser to prevent the lysis of protoplasts produced by the action of lytic enzymes. The effect of sucrose (0.5 M) on the lysis of lactic streptococci by phage lysin is shown in Table 3.10. It is apparent that a 0.5 M solution of sucrose did not inhibit the lysis of Group N streptococci by phage lysin.

Table 3.9

Effect of freeze-drying on the sensitivity of  
lactic streptococci to phage lysin

Strain	$\Delta$ O.D./min	
	Harvested cells	Freeze-dried cells
C2	0.30	0.773
RM1	0.218	0.329
SK11	0.173	0.318
US3	0.009	0.034

Stationary phase cells (1% inoculum, 22°C, 16 h) were harvested, washed and divided into 2 portions. One was suspended in buffer and tested for lysin sensitivity whilst the remaining portion was tested after freeze-drying as described in Methods.

Table 3.10

Effect of sucrose on the lysis of lactic  
streptococci by phage lysin

Sucrose concentration (M)	0	0.5
C2 ( $\Delta$ O.D./min)	0.39	0.42
SC1 ( $\Delta$ O.D./min)	0.50	0.50
1249 ( $\Delta$ O.D./min)	0.6	0.58

Stationary phase cells (1% inoculum, 22°C, 16 h) were suspended in 0.1 M phosphate buffer with or without sucrose and lysin sensitivity determined as in Methods.

## DISCUSSION

The results reported here demonstrate that several factors influence the sensitivity of Group N streptococci to  $\phi$ C2(W) lysin. Unlike lysozyme (Neujahr, 1966; Shockman *et al.*, 1968; Klaenhammer *et al.*, 1978) phage lysin lysed stationary as well as log phase cells. However, log phase cells were more sensitive than stationary phase cells. Chemical modification of the peptidoglycan as cells go into the stationary phase has been suggested as an explanation for the increased resistance of stationary phase cells to lysozyme. Resistance has been attributed to the presence of N-non-substituted glucosamine residues (Hayashi, Araki and Ito, 1973); a high degree of peptide cross-linking (Strominger and Ghuyssen, 1968); the presence of O-acetyl groups (Brumfitt *et al.*, 1958; Logardt and Neujahr, 1975); or the attachment to the peptidoglycan of polymers such as teichoic acid (Mandelstam and Strominger, 1961) or polysaccharide (Krause and McCarty, 1961). Further work will be required to determine which, if any, of these mechanisms are responsible for the increased resistance of stationary phase cells to phage lysin.

Several reports of autolytic activity in Group N streptococci have been published (Moustafa and Collins, 1968a; Moustafa and Collins, 1968b; McDonald, 1971; Mou, Sullivan and Jago, 1976). Synergism between autolysin activity and lysozyme has been suggested as a factor involved in the increased lysozyme sensitivity of log phase cells of *Str. faecalis* (Shockman *et al.*, 1968). The results of this study show that the sensitivity of *Str. lactis* C2 to lysin is highest in early log phase and decreases thereafter whereas highest autolytic activity was found with late log/early stationary phase cells. Thus autolytic activity does not appear to be a factor involved in the increased sensitivity of log phase cells of *Str. lactis* C2 to phage lysin. That autolytic

activity is higher in other Group N streptococci before transition to the stationary phase has been found by Mou *et al.* (1976). Synergism was not found to explain the increased sensitivity of log phase cells of *Lact. fermenti* to lysozyme (Neujahr *et al.*, 1973). The sensitivity of this organism to lysozyme peaks during the log phase, after autolytic activity has started to decline.

That cells grown in media containing glucose were more easily lysed by  $\phi$ C2(W) lysin than those grown on other substrates could have been anticipated from the results of Chassy and Giuffrida (1980) and the findings of Gasson (1980) and Klaenhammer *et al.* (1978) with lysozyme. The finding that cells grown on gluconate were relatively insensitive to lysin was unexpected and revealed that the energy source used in PLGYG broth had a major influence on lysin sensitivity. The mechanism whereby carbohydrate or carbohydrate-type substances influence lysin sensitivity is unknown. It is conceivable that cell wall structure and/or composition could be influenced.

Production of extracellular polymeric material by Group N streptococci has been reported (Forsen, 1966; Brooker, 1976; Sozzi *et al.*, 1978; Saxelin *et al.*, 1979) and could explain the differences in lysin sensitivity found with the different substrates if such material was induced by specific carbohydrates; this material could block the access of lysin to the cell wall. Forsen (1966) reported that capsule formation by lactic streptococci was optimal at 18°C or below. Since no significant difference in lysin sensitivity was found for cells grown on the different substrates at 22°C or 30°C with the exception of lactose, it is unlikely that the production of extracellular material is involved in the low sensitivity of cells grown on sugars other than glucose to lysin.

## lactic acid

Attempts to increase the sensitivity of <sup>l</sup>bacteria to lytic enzymes have not previously included growth in the presence of sub-lethal concentrations of penicillin G. This is surprising since it is well established that this agent inhibits terminal transpeptidation and results in peptidoglycan chains which are not cross-linked and lack tensile strength. The results of this study have shown that growth of lactic streptococci in media containing low concentrations of penicillin can markedly increase lysin sensitivity. Difficulty may be experienced in applying this method to strains of lactic streptococci which exhibit high sensitivity to penicillin G, e.g. the *Str. cremoris* strains used in this study (p205). If difficulties are experienced the author would advise that other agents which also inhibit cross-linking, e.g. D-cycloserine, bacitracin, vancomycin, ristocetin and the cephalosporins should also be evaluated for their potential to increase lysin sensitivity.

Threonine has also been reported to interfere with peptide cross-linking in the peptidoglycan (McCarron and Chang, 1975, cited by Chassy and Giuffrida, 1980). No increase in lysin sensitivity was found for cells grown in media containing threonine. Although supplementation of growth media with threonine was reported to increase the sensitivity of lactic acid bacteria to lysozyme (Chassy and Giuffrida, 1980) close scrutiny of these workers' results reveals that the effect of this amino acid is strain dependent; supplementation increased the resistance of three strains to lysis. It may be significant that data has not been published showing that threonine increases the sensitivity of lactic streptococci to lysozyme. In a study concerned with acetaldehyde formation, Lees and Jago (1976) found high levels of threonine aldolase activity in cell-free extracts of Group N streptococci. Although this enzyme, which cleaves threonine to form acetaldehyde and glycine, was found in

other lactic acid bacteria studied, lower enzyme activities were usually found. It is possible that the high threonine-aldolase activity of Group N streptococci may result in threonine supplementation of growth media being less effective in preventing peptide cross-linking than with other bacterial groups. Supplementation of media with lysine, however, did increase the sensitivity of lactic streptococci to phage lysin. Although Chassy and Giuffrida (1980) found a similar effect for lactic acid bacteria with lysozyme, the mechanism was not reported. Since lysine is a constituent of the peptidoglycan of lactic streptococci (Schleifer and Kandler, 1967) it is conceivable that a high concentration of this compound in the external environment could inhibit peptide cross-linking, perhaps by inhibiting enzymes involved in lysine addition to growing peptide chains.

Several workers have reported that the basal medium in which cells are grown can influence their sensitivity to lysozyme (Chassy, 1976; Klaenhammer *et al.*, 1978). This was also found for  $\phi$ C2(W) lysin. Freeze-drying markedly increased the sensitivity of Group N streptococci to lysin. The increase in sensitivity was shown not to be due to freezing and is probably a consequence of the drying process. That drying increased the sensitivity of lactic streptococci to phage lysin has also been found by Oram and Reiter (1965). With *E. coli* B, however, storage in the frozen state was required to produce cells of high sensitivity of phage T4 lysozyme (Tsugita *et al.*, 1968).

Hydrolysis of bacterial cell walls by hydrolytic enzymes frequently results in overt cellular lysis. In many instances cell wall removal is required without cellular lysis, e.g. protoplast fusion techniques and osmotic stabilisers such as sucrose must be added to lysis buffers. It is desirable therefore that a cell wall degrading enzyme should be



active in sucrose concentrations required for protoplast stabilisation. The phage lysin was not inhibited in 0.5 M sucrose, which has been widely used for this purpose. Lysozyme, however, is inhibited with sucrose concentrations of 0.25 M (Neujahr *et al.*, 1973).

These results strongly suggest that  $\phi$ C2(W) lysin should be of greater value than lysozyme for cell wall removal in lactic streptococci. Nevertheless, strain dependant differences in sensitivity were apparent with this agent (p154) as with lysozyme. The phage contamination present in enzyme preparations could pose problems in certain circumstances. However,  $\phi$ C2(W) does have a narrow host range and providing hosts for this phage are not grown in the laboratory the phage contamination present in lysin preparations should not present serious difficulties. In this context it is interesting to note that phage lysins have been used to liberate cell wall bound proteases in lactic streptococci (Thomas, Jarvis and Skipper, 1974).

SUMMARY

The effects of cultural and other factors on the sensitivity of *Str. lactis* C2 and some other lactic streptococci to  $\phi$ C2(W)-induced lysin were studied. Cells from the logarithmic phase were lysed at a faster rate than those from the stationary phase. The latter were lysed ultimately to a similar extent, however. Autolytic activity was not shown to contribute to lysin sensitivity; the growth stages at which cells showed highest lysin sensitivity and maximum autolytic activity were different. The carbohydrate source used had a marked effect on lysin sensitivity. Glucose yielded the most sensitive cells, and gluconate the least sensitive cells. Growth on media containing L-lysine, L-threonine and/or a mixture of both did not result in cells of markedly increased lysin sensitivity. Small increases in lysin sensitivity were found for cells grown on lysine. Growth in media containing sub-lethal concentrations (0.1-0.15 IU/ml) of penicillin G markedly increased the sensitivity of *Str. lactis* strains C2 and C10 and *Str. diacetylactis* DRC2 to lysin. The basal medium in which cells were grown also influenced lysin sensitivity. PLGYG broth yielded the most susceptible cells of C2 to lysis. M17 broth, however, yielded the most sensitive cells of DRC2 to lysin. Cells recovered from MRS broth were consistently less sensitive to lysis than cells obtained from other media. Incubation temperatures of 22°C or 30°C had no significant effect on lysin sensitivity. Marked increases in lysin sensitivity were found for freeze-dried cells. Frozen cells had a similar lytic rate to unfrozen controls. Lytic rate of  $\phi$ C2(W) lysin was apparently unaffected by using sucrose at a concentration of 0.5 M as an osmotic stabiliser.

## CHAPTER 4

ISOLATION AND EVALUATION OF THE PHAGE RESISTANCE OF  
LYSIN-RESISTANT MUTANTS OF GROUP N STREPTOCOCCI

## INTRODUCTION

The hazards of phage infection in cheese manufacture particularly in factories using single-strain starters are well known. One solution to this problem is the use of phage-resistant mutants. It is noteworthy that the use of 'phage-immune variants' was suggested in the first paper on lactic streptococcal phages (Whitehead and Cox, 1935) as a method for ultimately overcoming phage problems in cheesemaking.

All bacterial populations contain small numbers of spontaneously occurring phage-resistant mutants. The presence of phage-resistant mutants in populations of lactic streptococci has been reported. Variants ranging from 0-66.2 cfu per  $1 \times 10^6$  total cfu have been found by Prouty and Parisot (1957). Robertson (1960) found strains of lactic streptococci to contain phage-resistant mutants ranging from 1.5 to 1643 cfu per  $1 \times 10^6$  total cfu. Recently Marshall and Berridge (1976) found phage resistant variants ranging from 5 to 2735 cfu per  $1 \times 10^8$  total cfu in populations of Group N streptococci.

The inability of a phage to replicate on mutant bacteria may be due to a multitude of factors including loss of receptor sites (Adams, 1959) loss of specific DNA uptake sites (porins) on the cell membrane (Braun, 1978) and resistance of the cell wall to phage lysins (Brumfitt, 1960; Oram and Reiter, 1968; Reiter, 1973; Marshall and Berridge, 1976).

Little is known of the mechanisms involved in the penetration of Gram-positive bacteria by phage. Phage for Gram-positive bacteria first encounter a peptidoglycan structure to which other components such as teichoic acids and polysaccharides are covalently attached. It is generally accepted that DNA present in the phage head must first traverse the wall

before it can gain entry to the cytoplasm. Because many phages have lytic activity due to lytic enzymes allegedly contained in or on the tail, a role for phage tail lysin in penetration of the host wall to produce a channel through which DNA can travel to the membrane seems plausible. Evidence for the role of phage lysins in the infective process was presented by Brumfitt (1960). This worker reported that a mutant of *Micrococcus lysodeikticus* which was resistant to hen egg lysozyme (E.C.3.2.1.17) was also resistant to both phage tail lysin and phage. Brumfitt suggested that the resistance of the mutant to phage was due to the inability of the phage enzyme to make a channel through the cell wall to allow DNA ejection. Phage tail lysin may also be involved in the infection of lactic streptococci by phage. The receptor for  $\phi$ ML3 is located on the cell membrane (Oram and Reiter, 1968; Oram, 1971) and it has been suggested (Oram and Reiter, 1968) that access of this phage to its receptor site requires the prior penetration of the wall by the lytic enzyme associated with the tail. In view of the discovery of holes in the cell wall of a *Str. lactis* strain through which protrusions of the cell membrane were observed (Hurst and Stubbs, 1969) the cell membrane may already be available to phages for *Str. lactis*. In a recent study of seven phage-resistant mutants, Marshall and Berridge (1976) found that adsorption of phage homologous for the parent strain still occurred for three strains. Because of this observation the authors inferred that resistance was due to the inability of phage lysin to break down the cell wall and allow DNA introduction. As proof for this hypothesis the former authors offered the observation that the lysis-from-without of two of the mutants could not be induced by phage homologous to the parent.

Because of the apparent involvement of phage lysins in DNA introduction Reiter (1973) has suggested that lactic streptococci which are resistant to phage lysin should also be resistant to phage. Although definitive evidence for the role of phage lysins in DNA introduction is not available, it has been suggested that lysins are involved in the release of phage from infected cells (Ralston *et al.*, 1955; Jacob and Fuerst, 1958; Ralston *et al.*, 1961; Mukai *et al.*, 1967). Nevertheless there are phage-host systems in which phage-induced lysins have not been demonstrated (Markert and Zillig, 1965; Zinder, 1965). If phage lysins are involved in the exit of phage from infected bacteria then it can be argued that lysin-resistant bacteria should be phage-resistant even if lysins are not involved in DNA introduction. The mechanism of resistance in this case can be envisaged as an entrapment process in which intracellular phage production occurs but release of mature virus can not take place because the cell wall is resistant to lysis by phage lysins.

If lysin-resistant lactic streptococci are phage-resistant as suggested by Reiter (1973) their use may overcome potential problems associated with other types of phage-resistant mutants. When foreign DNA enters a cell, it is usually destroyed by the cells restriction system. Restriction involves the degradation of foreign DNA by host endonucleases. Because restriction provides protection against phage attack the development of strains, using genetic engineering techniques, has been suggested as one approach to producing phage-resistant starter strains (Erickson, 1980). Several workers have shown that restriction systems in lactic streptococci can be inactivated by holding cells at elevated temperatures, e.g. 40°C (Pearce, 1978; Sanders and Klaenhammer, 1980). Because of the heat sensitivity of these systems it is plausible that the heat treatments used in the scalding stage of some cheese varieties may render restriction systems inoperative and result in phage attack. Phage

resistance can also be produced by a mutation altering the nature of the receptor site. Such mutants occur readily in the lactic streptococci. Three such variants have been described recently (Marshall and Berridge, 1976). However, such variants may be sensitive to phages to which the parent strain was originally insensitive (Hunter, 1947; Collins, 1958).

In this section experiments were undertaken to isolate lysin-resistant lactic streptococci and to determine their sensitivity to phage. Apart from the possible industrial significance of this work, it was envisaged that the results obtained would be helpful in clarifying the role of phage lysins in the phage-induced lysis of Group N streptococci.

## MATERIALS AND METHODS

### CULTURES AND BACTERIOPHAGES

The species identity and the origins of the cultures and phage used have been described previously (p20 and p99).

### MEDIA AND PROPAGATION CONDITIONS

Strains were grown in PLGYG broth and maintained in this medium as described previously (p20) unless stated otherwise.

### ISOLATION OF VARIANTS OF LACTIC STREPTOCOCCI RESISTANT TO EITHER $\phi$ C2(W) LYSIN OR LYSOZYME

Partially purified lysin was obtained by ion-exchange chromatography (p125) and was subjected to ultrafiltration using the Millipore PSVP membrane (p129) to obtain a preparation with a reduced phage concentration. Lytic enzyme preparations were filter sterilised using a syringe mounted filter of pore size 0.45  $\mu$ m. Highly purified lysin preparations could not be used because the filters (cellulose acetate and polycarbonate) bound the enzyme present in such preparations. Plates containing 300 u/ml of lysin (actual lysin concentration) were prepared by adding 2 ml of filter sterilised lysin to 8 ml of molten PLGYG agar or glycerophosphate milk agar designated GMA (Limsowtin and Terzaghi, 1976) contained in a standard 8.5 cm diameter petri dish. The lysin was added to the petri dish just before the agar started to gel and the contents were carefully mixed. Care is required since the lysin is heat labile and exposure to high temperatures must be carefully controlled to avoid denaturation and loss of activity. Agar media containing sterile lysozyme (Sigma Chemicals) were prepared in a similar manner. All operations were performed in a laminar flow cabinet to minimise the possibility



of contamination occurring. Plates were air dried for 1 h in the laminar flow cabinet, with the air flow on, and the lids partially removed. At this time the plates of media were inoculated with 0.1 ml of a late stationary phase PLGYG culture. The inoculum was spread over the surface with a sterile 'hockey stick' and incubated at 30°C. In all experiments control plates, to which culture had not been added, were incubated under similar conditions to ensure that the lysozyme and phage lysin preparations were sterile and that contamination had not occurred. The plates containing lysin contained about  $5 \times 10^7$  -  $1 \times 10^8$  pfu/ml of  $\phi C2(W)$ .

#### SENSITIVITY OF VARIANTS TO $\phi C2(W)$ -INDUCED LYSIN

Isolated colonies were picked off agar plates containing phage lysin or lysozyme and were used to inoculate 9 ml of PLGYG. In some experiments the broth also contained 300 u/ml of phage lysin or lysozyme (500 or 1000  $\mu\text{g/ml}$ ). Broth media were incubated at 30°C until good growth was evident.

Most isolates, providing that they actually grew, demonstrated satisfactory growth after 14-20 h incubation but some colonies required longer incubation up to 72 h. When satisfactory growth had occurred 0.1 ml of active culture was used to inoculate a further 10 ml of PLGYG. In some experiments lysozyme or phage lysin was added to the broth to maintain the selective pressure and help reduce the possibility of back mutation to lysin sensitivity. After 6 h incubation at 30°C the cells were harvested and their sensitivity to lysin determined as described previously (p24) except that, in some instances, higher lysin concentrations were used. For comparison, control experiments were also performed to determine the sensitivity of the parent cultures, grown under similar conditions, to phage lysin.

## SENSITIVITY OF VARIANTS TO BACTERIOPHAGE

Immediately prior to centrifugation, 0.2 ml of culture was removed. The phage sensitivity of this culture was then determined (p23).

## CARBOHYDRATE FERMENTATION

Variants were grown in PLGYG broth and used to inoculate tubes of basal medium (p21) containing a range of carbohydrate-type substrates as described previously (p21). Tubes were examined at intervals over a 72 h period at 30°C for evidence of acid production.

## ANTIBIOTIC SENSITIVITY OF LYSIN-RESISTANT VARIANTS

A disc assay was used. Assay media were prepared by adding 0.1 ml of a late stationary phase culture to 10 ml of molten (47°C) PLGYG agar contained within a standard 8.5 cm diameter petri dish. The contents were carefully mixed and allowed to harden on a level surface. Aqueous solutions of polymyxin B, ampicillin, penicillin G, streptomycin sulphate and neomycin were prepared. Antibiotic assay discs (Whatmans; Maidstone, Kent) of diameter 6 mm were dipped into solutions containing antibiotics, shaken free of excess liquid, and applied to the surface of inoculated plates. Assay media were incubated at 30°C for 24 h. At this time the diameter of the inhibition zones, if present, was measured.

## ANTIBIOTICS

Streptomycin sulphate, neomycin, ampicillin and polymyxin B were obtained from Sigma. Penicillin G was obtained from BDH Ltd.

## RESULTS

### LYSIN SENSITIVITY OF PHAGE-RESISTANT VARIANTS OF *Str. lactis* C2

Single-strains of lactic streptococci have been shown to be heterogenous in the sense that they contain variants which differ in such respects as milk protein utilisation, carbohydrate utilisation and phage sensitivity (for a review see Lawrence *et al.*, 1978). That populations of lactic streptococci maintained at this laboratory also contained phage-resistant variants is apparent from the data shown in Table 4.1. The strains studied contained from <1 to 400 variants per  $2 \times 10^8$  cfu. If lysin-resistant variants are phage-resistant it is likely that lysin-resistant variants should exist in a population of phage-resistant mutants. This possibility was studied by growing 30 phage-resistant C2 variants in PLGYG broth and determining their sensitivity to  $\phi$ C2(W)-induced lysin. The variants were obtained from secondary growth arising on double agar plates of C2 which had previously shown confluent lysis and were checked for phage sensitivity. The mutants were resistant to  $\phi$ C2(W) and  $\phi$ ML3. No lysin-resistant isolates were found.

### USE OF PHAGE LYSIN AS A SELECTIVE AGENT FOR ISOLATING LYSIN-RESISTANT MUTANTS

The previous results suggested that the frequency of C2 variants which were both lysin and phage-resistant was low, if they existed at all, and attempts were made to use the phage-induced lysin as a selective agent to isolate lysin-resistant mutants.

### ISOLATION OF LYSIN-RESISTANT MUTANTS OF C2

Plates of PLGYG were prepared containing about 300 u/ml of  $\phi$ C2(W)-induced lysin using the method described on page 222. The agar contained

Table 4.1

Phage-resistant variants\* present in cultures of lactic streptococci

Strain	Number of variants in $1 \times 10^8$ cfu
C2	140
C7	200
SC1	400
SK11	<1
1957	400
992	27
1299	27
1022	2
1271	3
1249	400
986	10
1246	300
C1	200

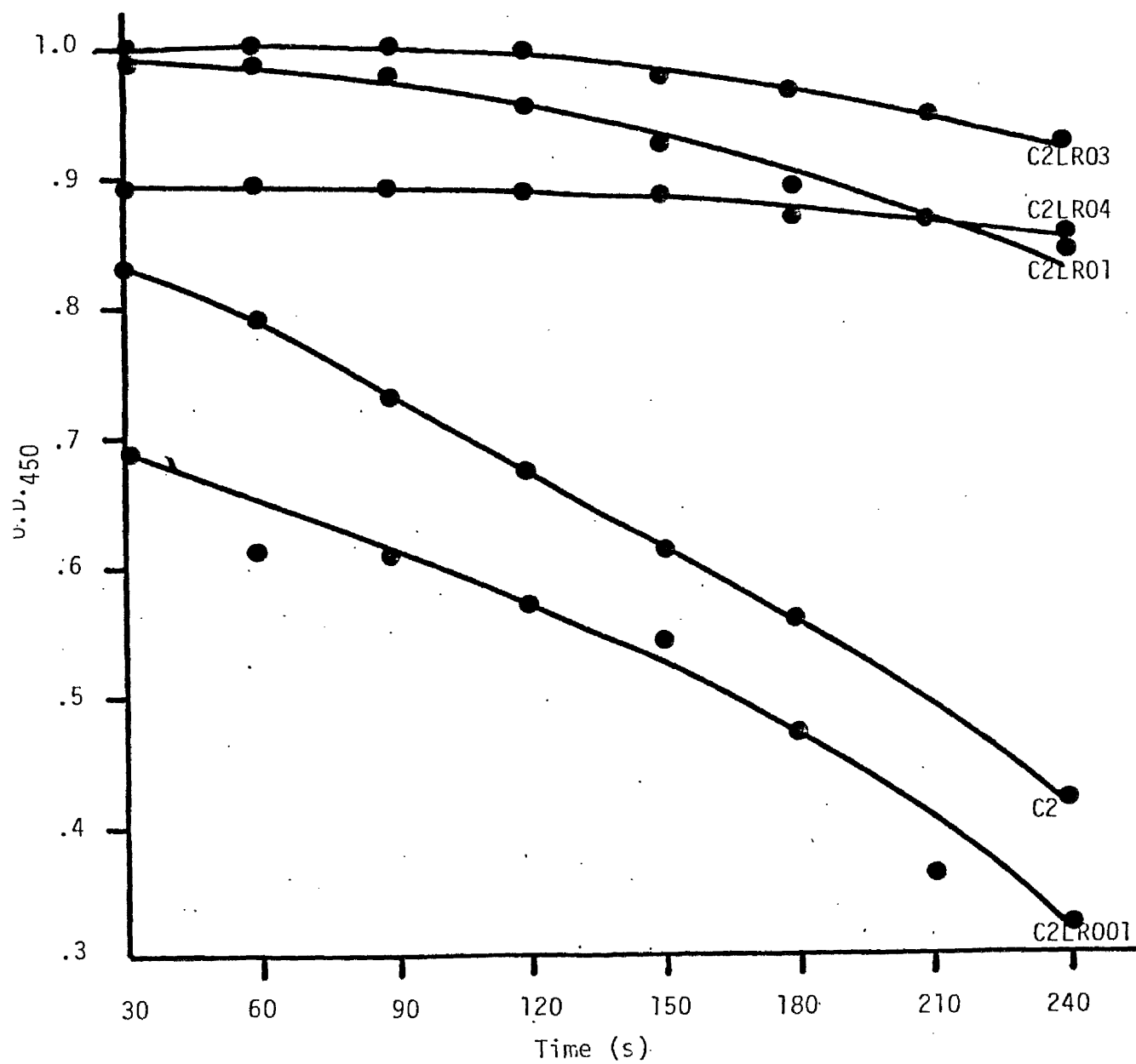
\*Resistant to their homologous phage.

about  $10^7$  -  $10^8$  pfu/ml of  $\phi C2(W)$ . In a series of experiments plates containing lysin were inoculated with about  $2 \times 10^8$  cfu of late stationary phase cultures of C2 which were spread over the surface of the plate with a sterile 'hockey stick'. In some experiments single colony isolates of C2 were also used. In some instances one to ten colonies were apparent after 24 h incubation at 30°C. However, at 48 h, colonies were usually present and ranged in number from 0-100 per plate. Similar results were obtained if a single colony isolate was used. Over a period of a year, about 200 colonies were picked off a range of plates and grown in PLGYG broth at 30°C. About 150 isolates exhibited good growth characteristics in PLGYG and were examined for sensitivity to lysin. In general all isolates tested were more resistant to lysis than the parent strain, however, some isolates were as sensitive to lysis, and in certain instances more sensitive than the parent strain. Typical data are presented in Figure 4.1. About 25 isolates exhibiting lysin response curves similar to C2LR04 were examined for sensitivity to  $\phi C2(W)$ ,  $\phi 712$  and  $\phi ML3$ . All the isolates tested failed to propagate  $\phi C2(W)$  or  $\phi ML3$ , but were sensitive to  $\phi 712$ . These results apparently suggest that lysin-resistant mutants of C2 do not propagate  $\phi C2(W)$  or  $\phi ML3$ . However, the lysin preparations contained high levels of  $\phi C2(W)$  and the possibility that the selection of both lysin and phage-resistant mutants was taking place simultaneously existed.

#### ISOLATION OF C2 MUTANTS RESISTANT TO HEN EGG LYSOZYME

In the absence of lysin preparations free from  $\phi C2(W)$  an attempt was made to find another lytic enzyme which had a similar action on cell walls to the  $\phi C2(W)$ -induced lysin. The phage enzyme is a glycosidase that appears to attack the muramic acid linkage in the peptidoglycan (p<sub>NA</sub>) and thus appears to have a similar specificity to hen egg lysozyme. If mutants of C2 could be isolated which were resistant to lysis by the hen egg enzyme

Figure 4.1 Action of partially purified  $\phi$ C2(W) lysin on variants of *Str. lactis* C2



and also resistant to  $\phi C2(W)$ -induced lysis then their phage sensitivity could be evaluated without the complication of selective effects which might operate if a lysis preparation containing  $\phi C2(W)$  were used. In preliminary experiments PLGYG plates were prepared containing 5, 10, 100, 500 and 1000  $\mu\text{g/ml}$  of sterile lysozyme respectively. In these experiments three plates for each enzyme concentration were inoculated with about  $2 \times 10^8$  cfu of late stationary phase C2 cells and incubated at  $30^\circ\text{C}$  for 72 h. At intervals, plates were examined for growth (Table 4.2). Confluent growth was present on all plates containing enzyme concentrations in the range 5-100  $\mu\text{g/ml}$  at 24 h. Colonies were present on one plate containing 500  $\mu\text{g/ml}$  of lysozyme at 24 h and by 72 h colonies were present on all three plates. At 72 h colonies ranged from about 30-100 (excluding pin-point colonies). On one plate, there were numerous pin-point colonies whose size did not increase with continued incubation. Colony numbers on the 1000  $\mu\text{g/ml}$  plates were always considerably lower than those on the plates containing 500  $\mu\text{g}$  of lysozyme. At 72 h colonies on the 1000  $\mu\text{g/ml}$  level plates ranged from 0-20. A range of colonies was picked off plates containing 500 and 1000  $\mu\text{g/ml}$  of hen egg lysozyme and grown in PLGYG broth. In most experiments the broth was supplemented with 500 or 1000  $\mu\text{g/ml}$  of lysozyme in order to eliminate the growth of sensitive variants which could arise from back mutation if the selective pressure was removed. In general colonies took 3-4 days to grow in PLGYG - many colonies failed to grow. Once growth had been initiated it was usually more rapid on transfer. Many isolates, however, failed to grow on the second subculture. Lysis sensitivity studies were performed on 6 h (1%,  $30^\circ\text{C}$ ) cultures. In general all isolates tested were less sensitive to  $\phi C2(W)$  lysis than the parent strain. On the basis of the lysis sensitivity data three variants were found which were virtually resistant to lysis (Figure 4.2). These variants were tested for sensitivity to  $\phi C2(W)$ ,  $\phi ML3$  and  $\phi 712$ . All variants propagated  $\phi C2(W)$ ,  $\phi ML3$  and  $\phi 712$ .

Table 4.2

Effect of lysozyme (E.C.3.2.1.17) addition to PLGYG agar<sup>(1)</sup>  
on the growth of *Str. lactis* C2

Growth at	Lysozyme concentration (mg/ml)				
	5	10	100	500	1000
24 h	CG	CG	CG	1+	-
28 h	CG	CG	CG	2+	1+
72 h	CG	CG	CG	3+	2+

(1) 3 plates for each concentration of enzyme were prepared and inoculated with about  $1 \times 10^8$  cfu.

CG confluent growth on all plates.

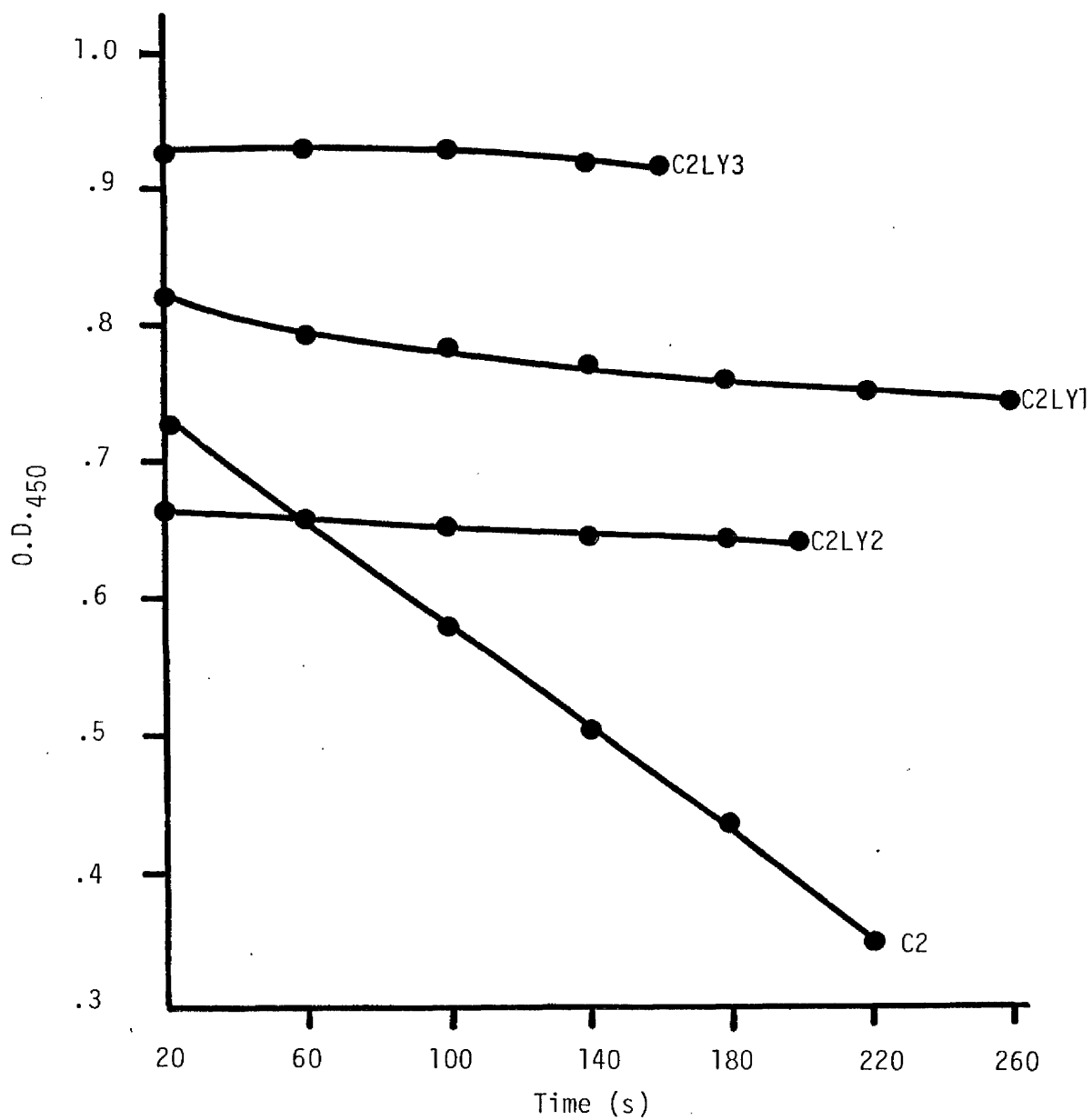
+ isolated colonies present; prefix gives number of plates showing growth.

- no growth.



Figure 4.2 Action of partially purified  $\phi$ C2(W) lysin on lysozyme (E.C.3.2.1.17) resistant variants of *Str.*

*lactis* C2



These results clearly show that lysin and phage resistance are not correlated for C2.

#### EFFECT OF GROWING *Str. lactis* C2 ON DIFFERENT CARBOHYDRATE SUBSTRATES ON THE TITRE OF $\phi$ C2(W)

It has been shown that certain carbohydrate containing media (p199) yield cells which are relatively resistant to lysin. This finding suggested that the phage sensitivity of cells grown in gluconate, for example, which yields cells relatively resistant to lysis compared with cells grown in glucose (sensitive to lysin) could provide further information concerning the relationship between lysin and phage sensitivity for C2. The results (Table 4.3) revealed that cells derived from the different substrates gave similar titres of  $\phi$ C2(W). These results also suggest that lysin and phage resistance are not correlated for C2.

#### ISOLATION OF LYSIN-RESISTANT MUTANTS OF *Str. lactis* C10

Further experiments were undertaken to obtain information concerning the relationship between lysin and phage sensitivity for other lactic streptococci.

PLGYG agar was prepared containing about 300 u/ml of lysin as described previously, and inoculated with about  $2 \times 10^8$  cfu of late stationary phase cells of C10. The inoculum was spread over the surface using a sterile right-angled glass rod. In some experiments single colony isolates were also used. Plates were incubated at 30°C and examined at intervals over a 72 h period. Colonies were rarely visible at 24 h, however, by 72 h at least one colony was present on one out of every six inoculated plates. At 72 h the number of colonies present on plates varied from 0-75. On some instances minute pin-point colonies were also found. Similar numbers of colonies were found in cultures obtained from single colony isolates. About

Table 4.3

Effect of growing *Str. lactis* C2 on different carbohydrate substrates on the titre of  $\phi$ C2(W)

Carbohydrate	pfu/ml
Glucose	$5 \times 10^9$
Gluconate	$3 \times 10^9$
Lactose	$4 \times 10^9$
Galactose	$5 \times 10^9$

Cells were grown in PLGYG broth in which glucose was substituted for the carbohydrate indicated. Late stationary phase cultures were then used to assay a  $\phi$ C2(W) preparation using PLGYG agar.

200 colonies were picked off lysin containing plates and grown in PLGYG broth at 30°C overnight (18 h). Lysin sensitivity tests were performed on washed 6 h log phase cells suspended in phosphate buffer. In general the isolates tested were more resistant to lysis by the phage enzyme than the parent strain. Variants were found which were relatively resistant to  $\phi$ C2(W)-induced lysin. Typical data are shown in Figure 4.3. It is apparent that several isolates were virtually lysin-resistant, e.g. C10-MB and C10-1012. Two variants C10-MA and C10-MC which gave lysin response curves similar to C10-MB and C10-1012 were unstable and failed to grow in PLGYG broth after two transfers. The sensitivity of the isolates to a range of phage is presented in Table 4.4. For comparison the lytic rate of the isolates is also presented. Of the 11 isolates tested seven retained sensitivity to the four typing phages. Amongst these isolates were the two variants (C10-03 and C10-MB) which were least sensitive to lysis by the phage enzyme. Two of the seven isolates gave a weak lytic reaction with  $\phi$ C10 and  $\phi$ ML8(1). Of the four remaining isolates, three (C10-1010, C10-1012, C10-1015) gave no lytic reaction with  $\phi$ SK3 but retained sensitivity to  $\phi$ C10,  $\phi$ ML8(1) and  $\phi$ ML8(W) and one (C10-1014) gave no lytic reaction with either  $\phi$ SK3 or  $\phi$ ML8(W) but retained sensitivity to both  $\phi$ C10 and  $\phi$ ML8(1). If the lysis rate of the isolate C10-1014 which has lost sensitivity to  $\phi$ ML8(1) and  $\phi$ SK3 is compared with that of other C10 variants which retained sensitivity to all four typing phages, e.g. C10-MB, C10-03, it is apparent that these latter strains are considerably more resistant to lysis. This finding suggests that lysin resistance and phage resistance are not correlated for *Str. lactis* C10.

The isolates were retested for sensitivity to the typing phages and similar results were obtained. The typing was also repeated with selected strains which had been grown for several transfers in PLGYG containing added

Figure 4.3 Action of partially purified  $\phi$ C2(W) lysin  
on variants of *Str. lactis* C10

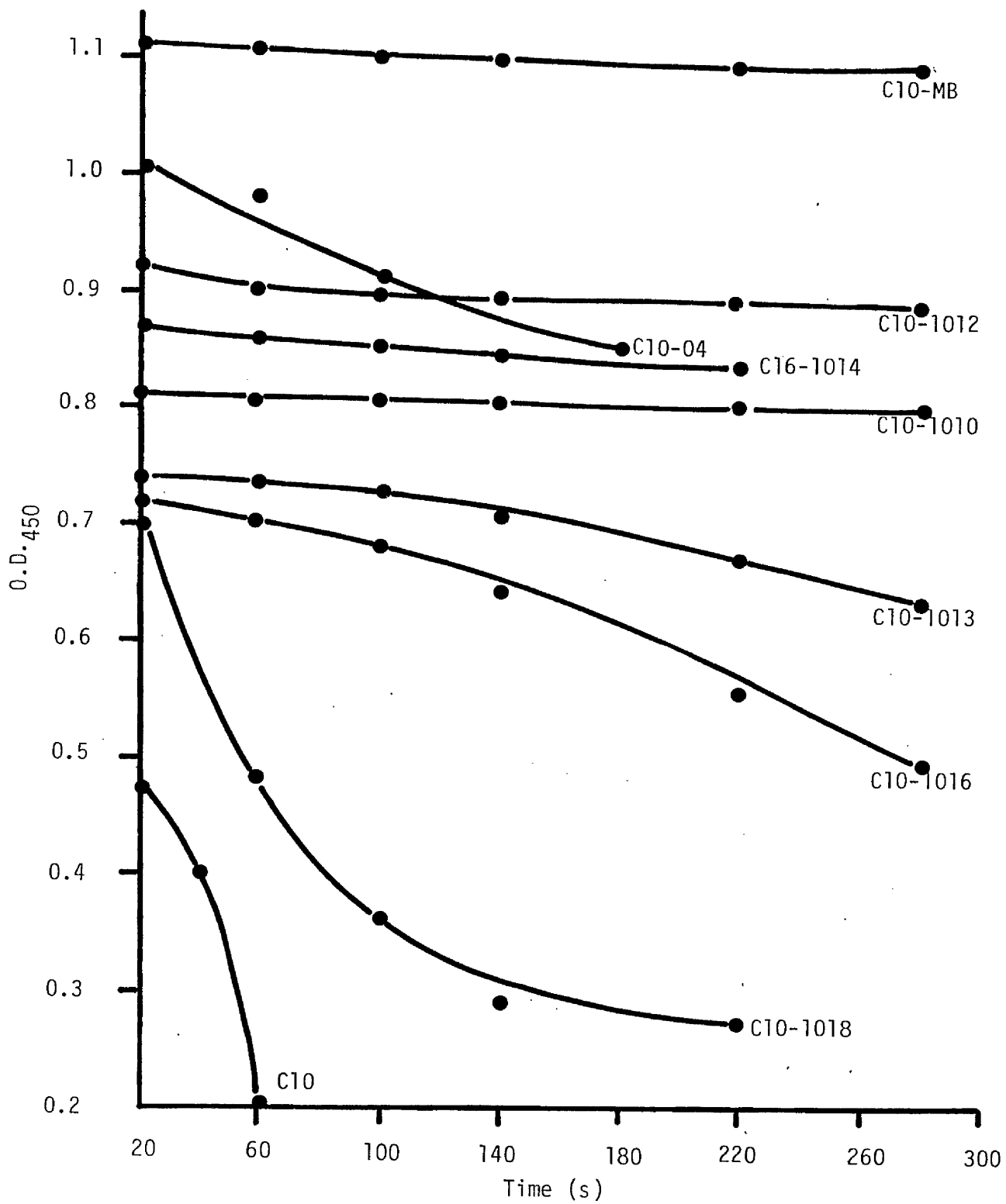


Table 4.4

Sensitivity of lysin-resistant variants of *Str. lactis* C10  
to bacteriophage

Strain	Lytic Rate <sup>(1)</sup> O.D./min	Phage					
		φC10	φML8(1)	φSK3	φML8(W)	φC2(W)	φ712
C10-1010	0.005	+	+	-	+	-	-
C10-1014	0.010	+	+	-	-	-	-
C10-1012	0.002	+	+	-	+	-	-
C10-1017	0.045	+	+	+	+	-	-
C10-1015	0.116	+	+	-	+	-	-
C10-1011	0.231	+	+	+	+	-	-
C10-1013	0.03	+	+	+	+	-	-
C10-1016	0.1	+	+	+	+	-	-
C10-03	0.004	+	+	+	+	-	-
C10-04	0.115	(+)	(+)	+	+	-	-
C10-MB	0.003	(+)	(+)	+	+	-	-
C10	1.2	+	+	+	+	-	-

(1) refer to Figure 4.1 for details.

a + sign indicates lysis; a - sign no lysis and (+) a weak lytic reaction.

lysin. The objective was to maintain the selective pressure and prevent back mutation to a sensitive variant. These experiments also yielded similar results to the previous experiments. In repeat experiments very slight zones of lytic activity were consistently obtained when  $\phi$ C10 and  $\phi$ ML8(1) were spotted onto double agar plates seeded with C10-MB and C10-04. These results suggested that phage-host interactions were occurring which were not revealed by the typing procedure used. In an attempt to obtain more information, C10-MB and C10-04 were used to titre both phage preparations (Table 4.5). For comparison several other variants were also studied. The explanation for the slight zones of lytic activity found with  $\phi$ C10 and  $\phi$ ML8(1) on C10-MB and C10-04 is apparent. Only very low phage titres were found with either strain. The practical significance of this finding is that C10-04 and C10-MB are less sensitive to  $\phi$ C10 and  $\phi$ ML8(1) than the other strains tested - hence the weak lytic reactions found. It is noteworthy that some of the other variants gave phage titres significantly different from the parent strain. Strain C10-1012 plated both phages more efficiently than C10. The situation was reversed for C10-1017.

The possibility that differences in phage adsorption could explain the failure of C10-04 and C10-MB to plate  $\phi$ ML8(1) and  $\phi$ C10 less efficiently than C10 was considered. Similar adsorption of  $\phi$ ML8(1) occurred to both C10-04 and C10, thereby disproving this hypothesis at least for  $\phi$ ML8(1) and C10-04 (Table 4.6). A reduced level of adsorption was found with C10-MB, however, this level of reduction would be unlikely to explain the lower EOP of  $\phi$ ML8(1) on C10-MB.

#### ISOLATION OF LYSIN-RESISTANT MUTANTS OF *Str. lactis* ML8

Attempts were made to isolate lysin-resistant variants of *Str. lactis* ML8 using  $\phi$ C2(W) lysin as a selective agent with the methods described

Table 4.5

Titres of  $\phi$ ML8(1) and  $\phi$ C10 on lysin-resistant mutants  
of *Str. lactis* C10

Strain	pfu/ml	
	$\phi$ C10	$\phi$ ML8(1)
C10-1017	$5 \times 10^6$	$1 \times 10^7$
C10-1012	$17 \times 10^6$	$13 \times 10^7$
C10-1014	$12 \times 10^6$	$6.8 \times 10^7$
C10-1010	$7 \times 10^6$	$6.4 \times 10^7$
C10-MB	$1.3 \times 10^2$	$1.8 \times 10^2$
C10-04	$1 \times 10^2$	$1.61 \times 10^2$
C10-P	$10 \times 10^6$	$4 \times 10^7$



Table 4.6

% Adsorption of  $\phi$ ML8(1) to lysin-resistant mutants  
of *Str. lactis* C10

Strain	% Adsorption
C10-MB	71
C10-04	97
C10	95

previously. It was considerably more difficult to obtain growth of ML8 variants than with C10 or C2. In most instances no colonies were observed on plates containing lysin after 72 h incubation at 30°C. On some plates 50-100 pin-point colonies were present. From previous observations of pin-point colonies on C10 and C2 plates it was considered that these colonies would be unlikely to grow in PLGYG. This was confirmed for a small number (five) of pin-point colonies. Eventually about ten colonies growing on agar containing lysin in the size range 0.5-1 mm (72 h, 30°C) were found, transferred into PLGYG broth and examined for sensitivity to lysin. A selection of the results obtained is shown in Figure 4.4. As with C2 and C10 most of the isolates were less sensitive to lysis than the parent and some isolates were more sensitive to lysis - ML8-09. The two variants found to be the least sensitive to  $\phi$ C2(W)-induced lysin (ML8-01, ML8-04) were not as resistant to lysis as many of the C10 and C2 variants tested. This does not necessarily mean that ML8 variants in general are less resistant to the phage lysin than the other strains tested since only a small number of ML8 variants were studied. The sensitivity of ML8-01 and ML8-04 to phage is shown in Table 4.7. Both variants retained sensitivity to the typing phages.

#### PROPERTIES OF LYSIN-RESISTANT VARIANTS

Since lysin-resistant variants of Group N streptococci have not been described previously attempts were made to partially characterise a limited number of C10 and C2 variants. The characterisation studies were connected with (a) antibiotic sensitivity; (b) fermentation of carbohydrates; (c) growth in RSM and (d) lysin stability.

Figure 4.4 Action of partially purified  $\phi$ C2(W) lysin on variants of *Str. lactis* ML8

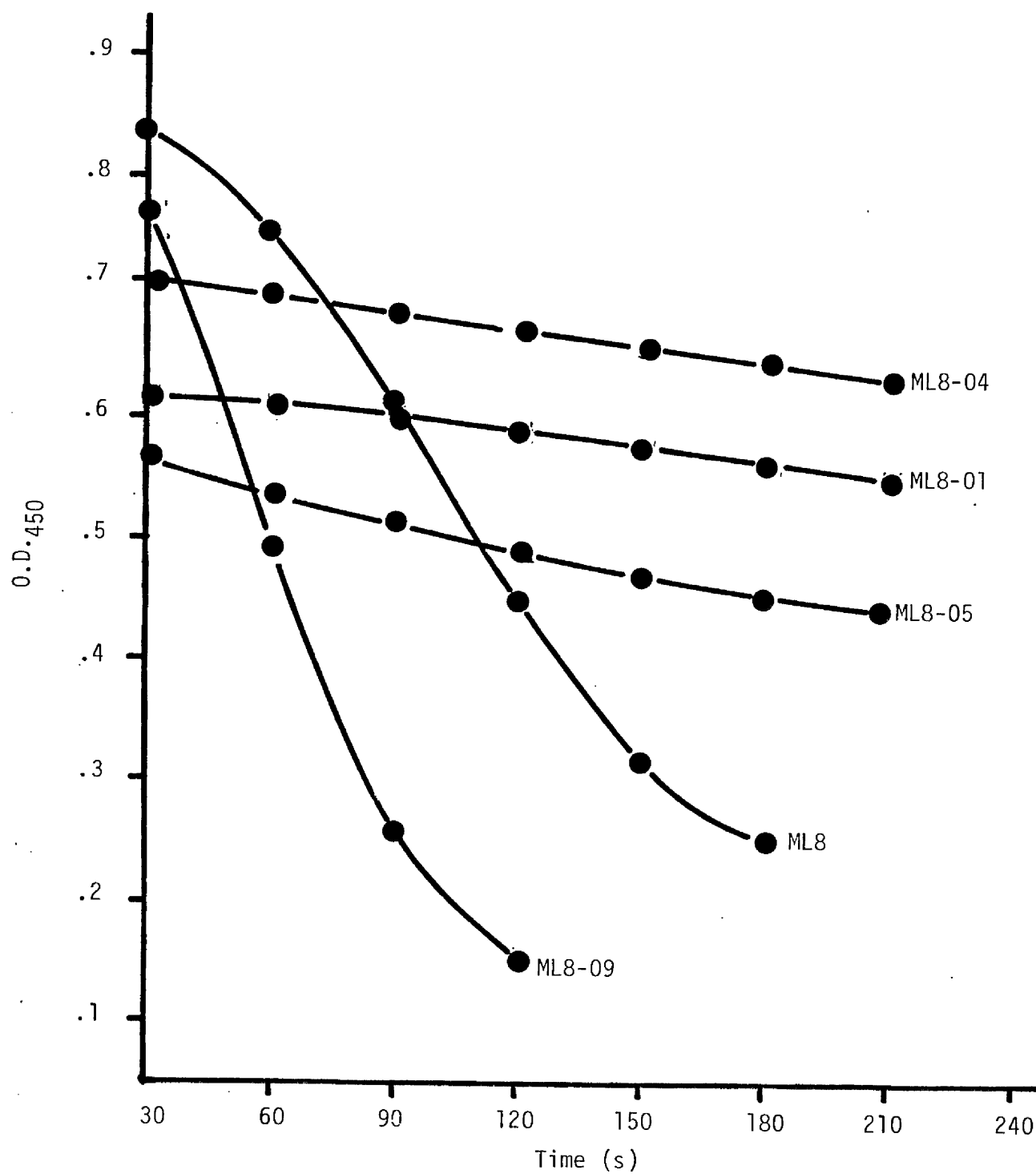


Table 4.7

Sensitivity of lysin-resistant variants of *Str. lactis* ML8  
to bacteriophage

Strain	Phage				
	$\phi$ ML8(1)	$\phi$ ML8(W)	$\phi$ SK3	$\phi$ WM1	$\phi$ C2(W)
ML8-01	+	+	+	+	-
ML8-04	+	+	+	+	-
ML8	+	+	+	+	-

a + sign indicates lysis of a host; a - indicates no lysis.

### Sensitivity to antibiotics

The sensitivity of the variants to polymyxin, ampicillin, penicillin G, streptomycin and neomycin was determined using a disc assay method. All strains tested gave no zone of inhibition with 50 IU of polymyxin and 1 IU of penicillin G. The results obtained are summarised in Table 4.8. Of the four C10 variants studied, three gave similar qualitative results for antibiotic sensitivity to the parent strain. C10-1010 was insensitive to 2.0 IU of penicillin G. If the magnitude of the diameter of the inhibition zone is accepted as providing a quantitative estimate of the sensitivity of a strain to an antibiotic, then differences in antibiotic sensitivity are apparent between the C10 variants and the parent culture. All the variants gave larger zones of inhibition with ampicillin, penicillin G (except C10-1010 which was resistant), streptomycin and neomycin than C10 suggesting that they were more sensitive to inhibition by these agents. This was particularly noteworthy for C10-1010 with neomycin.

Only two C2 variants were studied. Both variants gave similar results to the parent culture except C2-PL which had acquired sensitivity to 300 IU of polymyxin.

### Sugar fermentation

The ability of the variants to ferment a range of carbohydrates was determined (Table 4.9). All the variants with the exception of C2-1003 had similar carbohydrate fermentation patterns to the parent strains. C2-1003 failed to ferment maltose even on extended incubation.

### Growth in milk

For a lactic streptococcal strain to be of value in the dairy fermentation industry it must grow rapidly in milk. The ability of the

Table 4.8

Sensitivity of lysin-resistant variants to antibiotics

Antibiotic	Concentration <sup>(1)</sup>	Diameter of zone of growth inhibition (mm)								
		C10	C10-1014	C10-1010	C10-1012	C10-MB	C2	C2-1003	C2-PL	
Polymyxin	300	0	0	0	0	0	0	0	11	
Ampicillin	0.1	20	22	25	27	25	20	21	22	
Penicillin G	2.0	7	9	0	10	10	0	0	0	
Streptomycin	0.1	8	11	17	15	12	14	15	14	
Neomycin	0.1	11	13	14	15	13	12	13	14	

(1) concentration of polymyxin and penicillin G in international units; others in ug.

Table 4.9

Fermentation of carbohydrates by lysin-resistant mutants

Strain	Acid production from							
	Glucose	Fructose	Galactose	Gluconate	Maltose	Sucrose	Lactose	Mannitol
C10-1010	+	+	+	-(+)	+	-	+	+
C10-1014	+	+	+	-(+)	+	-	+	+
C2-1003	+	+	+	+	-	-	+	+
C10-MB	+	+	+	-(+)	+	-	+	+
C10-1012	+	+	+	-(+)	+	-	+	+
C2	+	+	+	+	+	-	+	+
C10	+	+	+	-(+)	+	-	+	+

+ acid production after 24 h.

-(+) slight acid production after 48 h.

- no growth or indicator change apparent at 72 h.

lysine-resistant mutants to grow in milk was determined by inoculating sterile RSM tempered to 30°C with a 1% inoculum of the respective variant. Growth was assessed by measuring the pH after 6 h incubation. In these experiments late stationary phase PLGYG broth cultures were used for inoculation rather than cultures which had been previously grown and acclimatised to milk. This practice was not possible because many of the variants would not grow in this medium. The results obtained are shown in Table 4.10. Of the four C10 variants, three produced little pH change in RSM at 6 h indicating that they did not grow well. The remaining variant, C10-M3 produced sufficient acid to give a pH of 5.65 showing that it grew fairly well in milk. However, none of the C10 variants grew as well as the parent strain which attained a pH of about 5.2 after 6 h incubation at 30°C.

One of the C2 variants also showed poor acid production in milk, giving a pH of about 6.3 after 6 h. The remaining strain, C2-1003, grew fairly well producing a pH of 5.65 at 6 h. This figure is lower (indicating more acid production) than the value of 5.73 obtained for the parent strain on this occasion. However, this value of 5.73 was not typical for C2. In previous and in repeat experiments in which C2 was grown in RSM prior to performing the activity test, this strain consistently gave 6 h pH values of less than 5.3. These results show that in general lysine-resistant variants grow poorly in milk. Nevertheless, two of the variants tested did grow fairly well suggesting that it might be possible to isolate lysine-resistant mutants which had similar acidification rates in milk to the parent strain.

Limsowtin and Terzaghi (1976) have described a medium designated GMA which permits the differentiation of fast and slow acid producing strains. GMA was prepared containing 300 u/ml of lysine and used to isolate C2 variants. The results of a typical experiment are shown in Table 4.11.



Table 4.10

Acid production by lysin-resistant mutants  
in reconstituted skim milk

Strain	pH <sup>(1)</sup> at 6 h
C2	5.73
C2-PL	6.27
C2-1003	5.65
C10	5.18
C10-1010	6.13
C10-MB	5.65
C10-1014	6.11
C10-1012	6.11

(1) RSM (10% TS) was inoculated with 1% culture and incubated at 30°C.

Table 4.11

Use of GMA containing phage lysin to isolate  
fast acid producing variants

Strain	Plate number	Number of variants and colony size		
		Large	Small	Pin-point
C2	1	5	23	-
	2	0	7	-
	3	0	2	12
C10	1	0	-	>50
	2	0	-	>50
	3	0	-	>50

Late stationary phase cells (about  $1 \times 10^8$  cfu) were spread onto GMA containing 300  $\mu$ /ml of phage lysin.

In the experiment shown, five colonies were obtained with a similar colony morphology to C2. When these colonies were inoculated into 3 ml of RSM coagulation of milk occurred by 18 h at 22°C. These findings are typical of fast acid producing strains. The sensitivity of the isolates to lysin was determined and two variants C2LRM1 and C2LRM2 were found which exhibited sensitivity curves similar to C10-1012 (Figure 4.3). Activity tests revealed that both isolates gave a lower pH in RSM after 6 h at 30°C compared with C2 (Table 4.12). These results indicate that the changes which confer lysin resistance to C2 do not necessarily result in mutants which have lost the ability to grow in milk at a fast rate.

#### Stability of lysin resistance in lysin-resistant mutants

If lysin-resistant mutants are to be of commercial value it is desirable that they should retain resistance in the absence of selection pressure. Most of the isolates tested lost lysin resistance and reverted to lysin sensitivity after three to five transfers (1% inoculum, 22°C, 18 h), however, one isolate C2PLRM1 retained sensitivity for 19 transfers at which point the experiment was discontinued.

Table 4.12

Acid production by lysin-resistant C2 variants  
isolated from GMA

Strain	pH* at 6 h
C2	5.25
C2LRM1	5.0
C2LRM2	4.95

\*for experimental details see Table 4.10.

## DISCUSSION

Populations of *Str. lactis* C2 and some other streptococci were shown to be heterogeneous in the sense that they contained phage-resistant variants. If lysin-resistant variants of C2 are phage-resistant then a proportion of phage-resistant variants would be expected to be lysin-resistant. Preliminary experiments with phage-resistant mutants of C2 failed to find lysin-resistant variants. Several explanations for these findings are plausible. The frequency of lysin-resistant mutants may be much lower than for other mutations and it might have been necessary to test larger numbers of mutants. Secondly a proportion of the variants studied may have been lysin-resistant but reverted to lysin sensitivity under the test conditions. This is unlikely since the test procedure used virtually precluded this possibility. Of course, it is also possible that resistance to lysis by  $\phi$ C2(W)-induced lysin and sensitivity to phage are not correlated.

Using phage lysin as a selective agent lysin-resistant variants of C2 were isolated. These mutants did not propagate  $\phi$ ML3 and  $\phi$ C2(W) but were sensitive to  $\phi$ 712. Two explanations for these results were considered. Firstly lysin-resistant cells of C2 were resistant to  $\phi$ ML3 and  $\phi$ C2(W) because the cell walls were resistant to degradation by the phage-induced lysins (both phage produce lysins) whereas  $\phi$ 712 used a different mechanism to obtain the exit of mature virions from infected cells. The second explanation concerned the high level of  $\phi$ C2(W) particles present in the lysin preparations which were used to select for lysin-insensitive variants. Because of the phage present, the possibility existed that the simultaneous selection of both phage and lysin-resistant mutants was occurring.  $\phi$ ML3 and  $\phi$ 712 differ in several respects including the location of their phage receptors on ML3, which is now known to be closely related

to C2 (Davies *et al.*, 1981). Oram and Reiter (1968) found that  $\phi 712$  has a receptor on the cell wall whereas  $\phi ML3$  adsorbs to the cell membrane. The independence of the genetic control of  $\phi ML3$  and  $\phi 712$  phage receptors was demonstrated using two phage-resistant mutants; both were resistant to  $\phi ML3$  but retained sensitivity to  $\phi 712$ . Thus the selection of mutants resistant to  $\phi ML3$  and sensitive to  $\phi 712$  would be compatible with the results of the former workers;  $\phi C2(W)$  may also adsorb to the cell membrane. Recently Gasson (Dr. M. Gasson, N.I.R.D.) informed the author of similar experiments (unpublished) in which he used  $\phi ML3$  lysin (Oram and Reiter, 1965) to isolate lysin-resistant variants of *Str. lactis* ML3 which is closely related to C2. Variants were found which were lysin resistant and also resistant to  $\phi ML3$  but not to  $\phi 712$ . Because his lysin preparation was contaminated with  $\phi ML3$  Gasson concluded that simultaneous selection of lysin and phage-resistant mutants was probably occurring.

Since lysozyme is a N-acetylmuramidase and  $\phi C2(W)$  lysin appears to have the same substrate specificity it was envisaged that mutants resistant to lysozyme would also be resistant to  $\phi C2(W)$  lysin. This hypothesis was confirmed and provided an opportunity to assess the phage sensitivity of lysin-resistant variants of C2 without the complication of selective effects which may have operated in previous experiments. Since lysozyme-resistant variants retained sensitivity to  $\phi C2(W)$  and  $\phi ML3$  it is highly probable that simultaneous selection of lysin and phage-resistant mutants occurred in the previous experiments. Further evidence that lysin sensitivity and phage resistance were not correlated for C2 was provided by the finding that the EOP of  $\phi C2(W)$  was not significantly different on cells grown on media containing different carbohydrate type substrates. It had been previously established (p199) that cells grown in gluconate were relatively resistant to lysis compared with cells grown on glucose for example.

Lysin-resistant mutants of C10 and ML8 were also isolated and were shown not to be universally phage-resistant as predicted by Reiter (1973). Certain C10 variants acquired resistance to one or two of the typing phages. This was unlikely to be connected with lysin resistance because two of the most insensitive strains to lysin still retained sensitivity to the four typing phages that the parent propagated. These findings probably reflect the variation in phage sensitivity which can exist in isolates from strains of lactic streptococci (Limsowtin *et al.*, 1978). Two C10 variants, C10-MB and C10-04, were particularly interesting since they plated two of the typing phages at low efficiency. These results may be due to the possession of modification/restriction systems. The presence of modification/restriction systems operating within variants of the same lactic streptococcal strain has been reported (Limsowtin *et al.*, 1978).

The results of this study have shown that the lysin-insensitive strains of *Str. lactis* are not resistant to phage and disprove the hypothesis of Reiter (1973) that such strains should be resistant to phage.

If lysins are involved in the release of phage from infected bacteria as discussed in the Introduction, why do lysin-resistant streptococci retain sensitivity to phage? Lysin production may not be essential for either cell wall penetration nor the release of phage from infected cells. Since phage-induced lytic enzymes have not been found in an *E. coli*  $\phi$ X174 system by Markert and Zillig (1965), or in a *Corynebacterium diphtheriae* system by Natkin (1962) or in various RNA phage-host systems (see the review by Zinder, 1965), lysin production is apparently not essential for penetration and multiplication, at least in some phage-host systems. Since virtually no lytic activity was found in  $\phi$ 712 lysates by Oram and Reiter (1965) or by the author it is not inconceivable that lysins are not involved in the exit of  $\phi$ 712 from infected cells.

After studying the kinetics of endolysin (a phage-induced lysin) in three induced lysogenic strains of *E. coli*, Groman and Suzuki (1963) found that endolysin production seemed to correlate well with the kinetics of phage release. One of their strains, however, displayed lysis inhibition at 44°C although lysing normally at 37°C. Because endolysin accumulated under conditions of lysis inhibition they questioned whether the production of phage-induced lytic enzymes are an absolute requirement for the release of phage. The authors suggested that a change in membrane permeability which would alter the osmotic stability and result in cell lysis might be a common feature in all cases of phage-induced lysis.

Groman and Suzuki (1966) attempted to test their hypothesis by adding spermine to cultures of *E. coli* infected with three different phages: a double stranded DNA phage -  $\lambda$  C50, a single stranded DNA phage -  $\phi$ X174 and a double stranded RNA phage -  $f_2$ . Spermine is a polyamine which can stabilise protoplasts and spheroplasts, including those of *Str. lactis* strains, in distilled water (Tabor, 1962; Grossowicz and Ariel, 1963; Groman and Suzuki, 1966; Erskine, 1970). Spermine stabilised two of the phage-host systems, the  $\phi$ X174 and  $f_2$  systems at concentrations ranging from  $2 \times 10^{-3}$  to  $4 \times 10^{-2}$  M but failed to stabilise  $\lambda$ -infected cells at concentrations up to  $8 \times 10^{-2}$  M. Stabilisation was shown by the use of optical density measurements and in the retention of mature phages in structures (presumably infected bacteria) sedimentable at low speed. These results, as the authors acknowledged, failed to completely support their hypothesis, but suggest that membrane permeability changes may be involved in the release of phage from, at least, some infected cells.

Erskine (1970) reported that the addition of spermine to cultures of *Str. lactis* ML3 infected with phage protected the cells from lysis. Although Groman and Suzuki (1966) found that this agent stabilised phage-



infected cells by preventing the release of mature virions from osmotically stabilised cells Erskine (1970) could find no evidence that mature virions were present or that the lysis inhibition effect was due to osmotic stabilisation.

It is also possible that a lytic agent other than  $\phi$ C2(W) lysin is present in  $\phi$ C2(W) lysates and it is this agent which promotes cellular lysis and release of phage from *Str. lactis* C2. If this enzyme has a different specificity than  $\phi$ C2(W) lysin, e.g. if the glucosamine linkage is attacked or the peptide portion of the peptidoglycan is hydrolysed the changes which confer resistance to  $\phi$ C2(W) lysin may not then result in resistance to lysis. However, no evidence for another lytic enzyme was obtained in crude lysates or lysates concentrated by ultrafiltration and fractionated by gel filtration. In this context it must also be noted that only one type of lytic agent was found in phage lysates of lactic streptococci by Oram and Reiter (1965) or Tourville and Tokuda (1967). In addition the finding by Goepfert and Naylor (1967) that a phage-induced peptidase type enzyme failed to lyse lysozyme-resistant cells suggests that cross resistance to lytic enzymes can occur. The probability of such an enzyme existing is therefore low.

Data has been presented in Chapter 2 concerning the failure to demonstrate lytic activity with high titre preparations of  $\phi$ C2(W) or to isolate a lytic enzyme from particles of  $\phi$ C2(W). These findings combined with the phage sensitivity of lysin-resistant streptococci are consistent with a dispensable role of  $\phi$ C2(W) lysin in the infection and release of  $\phi$ C2(W) particles from infected cells of *Str. lactis* C2.

If phage-induced lytic enzymes are dispensable in the release of mature virions from infected cells it is necessary to reconsider the findings of Brumfitt (1960) and Marshall and Berridge (1976).

Brumfitt (1960) reported that a mutant of *M. lysodeikticus* (LR1) which was resistant to lysozyme failed to propagate phages N1 and N4. In view of the results obtained with the C10 variants, the simplest explanation for Brumfitt's results is that his mutant was resistant to phage for some other reason than the inability of phage tail lysin to degrade the cell wall and permit DNA ejection. It is unfortunate that LR1 was not tested against other phages to determine if it was universally phage resistant. Secondly it is regrettable that Brumfitt did not isolate other lysozyme-resistant mutants to determine whether phage-insensitivity was truly a property of such mutants. In the absence of this information the author suggests that Brumfitt's results can be explained by his chance selection of a mutant that was both lysozyme and phage resistant.

Since Marshall and Berridge (1976) used phage to select for phage-resistant variants it is probable that simultaneous selection of both lysin and phage-resistant cells could have occurred.

If lytic enzymes are not essential to the lytic process it is valid to consider what value the ability of a phage to induce lysin-production by infected host cells has to the virus. Groman and Suzuki (1963) speculated that lysin production has a selective value for a phage since the ability to destroy the cell wall could result in a more efficient release and dispersal of phage. If this is accepted as a working hypothesis then lysin-producing phages would be expected to have a markedly faster replication rate, due to a reduced latent period perhaps, compared with non-lysin producers. Since the latent period and burst size of  $\phi$ C10-1 and

$\phi$ C10-11 (both produce lysin) and  $\phi$ C10-111, which does not produce lysin, are similar (Keogh, 1973) and the replication rates of  $\phi$ C2(W) and  $\phi$ 712 in broth are also similar (Figures 1.9 and 1.10) the data available for Group N streptococcal phages do not support Groman and Suzuki's hypothesis.

Although the lysin-resistant variants studied by the author propagated phage, the use of lysin-insensitive strains in paired or multi-strain cultures may give rise to cultures of increased resistance to phage infection; if a component strain was infected with a lysin-producing phage the remaining strains should continue to produce acid provided that they are insensitive to lysin. In this context the data presented in Chapter 1 showed that such an approach can result in cultures of increased resistance. Lysin-insensitive strains may be obtained by selecting spontaneous mutants from cultures of lactic streptococci or by selecting established strains which are lysin insensitive, e.g. AM2, US3, or DRC2. If mutation is used the culture selected should grow in milk at a fast rate and lysin resistance should be stable to repeated sub-culturing.

Lysozyme and phage-resistant cells of *M. lysodeikticus* were found to have a reduced growth rate compared with the parent (Brumfitt, 1960). Because of this observation this worker suggested that the changes which confer resistance to lysozyme (in this case acetalation of -OH groups) result in cells with an impaired growth capability. Although the majority of lysin-resistant streptococci selected at random in this study had a reduced acidification rate in RSM compared with the parent strain, fast acid producing variants were isolated using GMA. This finding shows that the changes in cell wall composition which result in resistance to phage lysin do not necessarily impair growth and acid production in milk. Marshall and Berridge (1976) described two variants which were resistant to lysis-from-without and which grew at a fast rate in milk. If it is accepted that

the failure of these mutants to lyse was due to a change in cell wall composition which resulted in cells resistant to the phage tail lysin then this report can be accepted as additional evidence that lysin resistance does not necessarily result in a reduced growth response in milk.

Phage-resistant variants of lactic streptococci have frequently been shown to be unstable and to revert to phage sensitivity (Whitehead and Cox, 1936; Czulak and Naylor, 1956; Lawrence *et al.*, 1976; Lawrence *et al.*, 1978) presumably due to back mutation. Although the majority of the lysin-resistant variants were unstable and lost resistance to lysin readily, one mutant maintained resistance for 19 transfers suggesting that it may be possible to isolate stable lysin-resistant variants.

In general the antibiotic sensitivity and the ability to ferment carbohydrates of the variants were similar to the parents. Milk frequently contains antibiotics (Walker *et al.*, 1981). Because of the increased sensitivity of some variants to certain antibiotics it would be desirable to test all lysin-resistant variants for antibiotic sensitivity prior to use as cheese starters. It is interesting that one of the procedures used in New Zealand to select replacement strains for cheesemaking is sensitivity to antibiotics (Lawrence *et al.*, 1978).

It is of interest to speculate on the changes in cell wall composition which could result in resistance to lysis from phage lysin. Data is available describing the cell wall modifications that result in resistance to lysozyme. Since lysozyme and  $\phi$ C2(W) lysin both appear to be n-acetylmuramidases the changes which confer resistance to lysozyme probably also give resistance to the phage lysin. Support for this hypothesis comes from the finding that C2 variants which were resistant to lysozyme were also resistant to phage lysin. Resistance to lysis by lysozyme has been attributed to acetylation of cell wall hydroxyl groups (Brumfitt *et al.*,

1958; Brumfitt, 1960); the presence of N-non-substituted glucosamine residues (Hayashi *et al.*, 1973); a high degree of peptide cross-linking (Strominger and Ghuyssen, 1968) or the attachment to the peptidoglycan of polymers such as teichoic acid (Mandelstam and Strominger, 1961) or polysaccharide (Krause and McCarty, 1961).

### SUMMARY

Experiments were undertaken to isolate variants of *Str. lactis* C2 and some other streptococci which were resistant, or relatively insensitive, to lysis by  $\phi$ C2(W)-induced lysin and to determine the phage sensitivity of any variants found. Using phage lysin as the selective agent, lysin resistant variants of C2 were isolated. Such variants occurred at low frequency ( $<1-100$  cfu per  $2 \times 10^8$  cfu) and were resistant to  $\phi$ C2(W) and ML3 but retained sensitivity to  $\phi$ 712. Because the selective agent also contained particulate phage, the possibility that simultaneous selection of lysin and phage resistant variants was occurring existed. The finding that lysozyme (E.C.3.2.1.17) resistant variants of C2 were also resistant to  $\phi$ C2(W)-induced lysin enabled the evaluation of the phage sensitivity of lysin resistant mutants without the complication of the relative effects which may have operated when phage lysin was used. The lysozyme and phage lysin-resistant variants retained sensitivity to  $\phi$ C2(W),  $\phi$ ML3 and  $\phi$ 712 showing that lysin-resistant mutants were not universally phage resistant. That lysin-resistant variants were not necessarily phage resistant was also confirmed for variants of *Str. lactis* C10. The significance of these results in evaluating the role of  $\phi$ C2(W)-induced lysin in the life cycle of  $\phi$ C2(W) is discussed.

Partial characterisation of some lysin-resistant variants with respect to antibiotic sensitivity, fermentation of carbohydrates, growth in RSM and stability of lysin resistance was undertaken. Although the majority of lysin-insensitive variants had a considerably reduced ability to grow in RSM, fast acid producers could also be isolated using glycerophosphate milk agar. The majority of variants were unstable and reverted, after several transfers, to lysin sensitivity. One variant, however, retained resistance to  $\phi$ C2(W) lysin for 19 transfers (1% inoculum,

16 h, 22°C) in RSM at which point the experiment was stopped. In general the sensitivity of variants to antibiotics and their ability to ferment carbohydrates were similar to the parent strains. The use of lysin-insensitive strains in paired and multi-strain cultures for cheesemaking is discussed.

# LITERATURE CITED

- Adams, M. H. (1959). *Bacteriophages*. New York: Interscience Publishers Inc.
- Andrews, J. (1964). Estimation of the molecular weights of proteins by Sephadex gel-filtration. *Biochem. J.* 9, 222-223.
- Anderson, E. B. and Meanwell, L. J. (1942). The problem of bacteriophage in cheesemaking. I. Observations and investigations on slow acid production. *J. Dairy Res.* 13, 58-72.
- Anon. (1975). *A laboratory manual of gel chromatography*. Bio-Rad Laboratories, 32nd and Griffin Ave., Richmond, California 94804, U.S.A.
- Anon. (1979). *Gel filtration theory and practice*. Pharmacia Fine Chemicals A.B., Box 175, S-75104 Uppsala 1, Sweden.
- Archibald, A. R., Ellwood, D. C. and Thomas, T. D. (1978). Bacteriophage propagation in chemostat cultures of *Streptococcus lactis*. *FEMS Microbiol.* 3, 339-342.
- Ashton, T. R. (1963). Cultured milk products. *J. Soc. Dairy Technol.* 16, 68-84.
- Babel, F. J. (1946). Factors influencing acid production by cheese cultures. II. Influence of bacteriophage on acid production in the manufacture of Cheddar and Cottage cheese. *J. Dairy Sci.* 29, 597-606.
- Babel, F. J. (1957). A low calcium medium for propagation of lactic cultures. *J. Dairy Sci.* 40, 604 (Abstract).
- Babel, F. J. (1962). Symposium on lactic starter cultures. V. Industrial utilisation of lactic cultures. *J. Dairy Sci.* 45, 1286-1290.
- Babel, F. J. (1977). Antibiosis by lactic culture bacteria. *J. Dairy Sci.* 60, 815-821.
- Barksdale, L. and Arden, S. B. (1974). Persisting bacteriophage infections, lysogeny, and phage conversions. *Ann. Rev. Microbiol.* 28, 265-299.
- Barkulis, S. S., Smith, C., Botralik, J. J. and Heyman, H. (1964). Structure of streptococcal cell walls. IV. Purification and properties of streptococcal phage muralysin. *J. Biol. Chem.* 239, 4027-4033.
- Bartell, P. F., Orr, T. E. and Lam, G. K. H. (1966). Polysaccharide depolymerase associated with bacteriophage infection. *J. Bacteriol.* 92, 56-62.
- Bleiweis, A. S. and Zimmerman, L. N. (1961). Formation of two types of osmotically fragile bodies from *Streptococcus faecalis* var. *liquefaciens*. *Can. J. Microbiol.* 7, 363-373.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254.



- Bradley, D. E. and Kay, D. (1960). The fine structure of bacteriophages. *J. gen. Microbiol.* 23, 553-563.
- Bradley, D. E. (1967). Ultrastructure of bacteriophages and bacteriocins. *Bacteriol. Revs.* 31, 230-314.
- Braun, V. (1978). Structure-function relationships of the Gram-negative bacterial cell envelope. In *Relations between structure and function in the prokaryotic cell*. ed. Stainier, R. Y., Rogers, H. J. and Ward, J. B. London: Cambridge University Press.
- Breen, J. N. (1969). Starter problems in Cheddar cheese. *N.Z. J. Agric.* 119, 74-77.
- Brem, S. P. and Welker, N. E. (1974). Characterization of a bacteriophage-induced, host specific lytic enzyme from lysates of *Bacillus stearothermophilus* infected with bacteriophage TP-8. *J. Bacteriol.* 120, 748-758.
- Bronfenbrenner, J. and Muckenfuss, R. S. (1927). Studies on the bacteriophage d'Herelle. VIII. The mechanism of lysis of dead bacteria in the presence of bacteriophage. *J. expt. Med.* 45, 887-892.
- Brooker, B. E. (1976). Cytochemical observation on the extracellular carbohydrate produced by *Str. cremoris*. *J. Dairy Res.* 43, 283-290.
- Brumfitt, W., Wardlaw, A. C. and Park, J. T. (1958). Development of lysozyme-resistance in *Micrococcus lysodieticus* and its association with increased O-acetyl content of the cell wall. *Nature (London)* 181, 1783-1784.
- Brumfitt, W. (1960). Studies on the mechanism of adsorption and penetration by bacteriophage. *J. Path. Bact.* 79, 1-9.
- Chassy, B. M. (1976). A gentle method for the lysis of oral streptococci. *Biochem. Biophys. Res. Commun.* 68, 603-608.
- Chassy, B. M. and Giuffrida, A. (1980). Method for the lysis of gram-positive, asporogenous bacteria with lysozyme. *Appl. Environ. Microbiol.* 39, 153-158.
- Chazaud, M. T. (1978). Etablissement de profils biochimiques pour les streptocoques lactiques: Importance pour les levains industriels. *Revue Laitiere Francaise* 361, 77-80.
- Cherry, W. B. and Watson, D. W. (1949). The *Streptococcus lactis* host-virus system. II. Characteristics of virus growth and the effect of electrolytes on virus adsorption. *J. Bacteriol.* 58, 611-620.
- Chopin, M. C., Chopin, A. and Roux, C. (1976). Definition of bacteriophage groups according to their lytic action on mesophilic lactic streptococci. *Appl. Environ. Microbiol.* 32, 741-746.
- Christensen, V. W. (1972). Recent developments in starter techniques. *Dairy Industries* 37, 655-659.
- Cleveland, R. F., Hölftje, A. J., Wicken, A. J., Thomasz, A., Daneo-Moore, L. and Shockman, G. D. (1975). Inhibition of bacterial wall lysins by lipoteichoic acids and related compounds. *Biochem. Biophys. Res. Commun.* 67, 1128-1135.

- Cleveland, R. F., Wicken, A. J., Daneo-Moore, L. and Shockman, G. D. (1976). Inhibition of wall autolysis in *Streptococcus faecalis* by lipoteichoic acids and lipids. *J. Bacteriol.* 126, 192-197.
- Collins, E. B. (1952a). Action of bacteriophage on mixed strain starter cultures. I. Nature and characteristics of the 'nascent phenomenon'. *J. Dairy Sci.* 35, 371-380.
- Collins, E. B. (1952b). Action of bacteriophage on mixed strain starter cultures. 2. Relation to acid production of the proportion of resistant bacteria. *J. Dairy Sci.* 35, 381-387.
- Collins, E. B. (1955). Action of bacteriophage on mixed strain cultures. IV. Domination amongst strains of lactic streptococci. *Appl. Microbiol.* 3, 141-144.
- Collins, E. B. (1958). Changes in the bacteriophage sensitivity of lactic streptococci. *J. Dairy Sci.* 41, 41-48.
- Collins, E. B. (1962). Behaviour and use of lactic streptococci and their bacteriophages. *J. Dairy Sci.* 45, 552-558.
- Crawford, R. J. M. and Galloway, J. H. (1962). Bacteriophage contamination of mixed-strain cultures in cheesemaking. *XVI Int. Dairy Congr. C*, 785-792.
- Crawford, R. J. M. and Galloway, J. H. (1964). Testing milk for antibiotic residues. *Dairy Industries* 29, 256-262.
- Crawford, R. J. M. (1972). Starter problems in the United Kingdom. *Dairy Industries* 37, 648-654.
- Czulak, J. and Naylor, J. (1956). Host-phage relationship of cheese starter organisms. III. Significance in selection and maintenance of starter cultures in commercial use. *J. Dairy Res.* 33, 131-133.
- Czulak, J., Bant, D. J., Blyth, S. C. and Crace, J. B. (1979). A new cheese starter system. *Dairy Industries* 44(2), 17-19.
- Daniell, S., Sandine, W. E. and Burningham, G. (1979). Commercial use of a multiple strain starter of known composition in Cheddar cheese manufacture. *J. Dairy Sci.* 62 (Suppl. 1), 70 (Abstract).
- Davies, F. L. and Gasson, M. J. (1981). Reviews of the progress of dairy science: genetics of lactic acid bacteria. *J. Dairy Res.* 48, 363-376.
- Davies, F. L., Underwood, H. M. and Gasson, M. J. (1981). The value of plasmid profiles for strain identification in lactic streptococci and the relationship between *Streptococcus lactis* 712, ML3 and C2. *J. Appl. Bacteriol.* 51, 325-337.
- Deibel, R. H. and Seeley, H. W. (1974). Streptococcaceae fam. nov. In *Bergey's Manual of Determinative Bacteriology* 8th edition. ed. Buchanan, R. E. and Gibbons, N. E. Baltimore: Williams and Williams.
- de Man, J. C., Rogosa, M. and Sharpe, E. (1960). Medium for the cultivation of *Lactobacilli*. *J. Appl. Bacteriol.* 23, 103-135.
- Dische, Z. (1962). Colour reactions based on the reducing properties of sugars. *Methods of carbohydrate chemistry* 1, 512-514.

- Dixon, M. and Webb, E. C. (1964). *Enzymes*. London: Longmans, Green and Co. Ltd.
- Doughty, C. C. and Hayashi, A. (1962). Enzymatic properties of a phage-induced lysin affecting group A streptococci. *J. Bacteriol.* 83, 1058-1068.
- Douglas, J., Qanber-Agha, A. and Phillips, V. (1974). Medium for the propagation and assay of lactic and other phages. *Lab. Practice* 23, 3-5.
- Efstathiou, J. D. and McKay, L. L. (1976). Plasmids in *Streptococcus lactis*: evidence that lactose metabolism and proteinase activity are plasmid linked. *Appl. Environ. Microbiol.* 32, 38-44.
- Eitenmiller, R. R., Friend, B. A. and Shahani, K. M. (1975). Relationship between composition and stability of bovine milk lysozyme. *J. Dairy Sci.* 59, 834-839.
- Elliker, P. R. (1950). The problem of bacteriophage in the dairy industry. *Proc. 11th Annu. Biol. Colloq. Oregon State University*.
- Emrich, J. and Stressinger, G. (1968). The role of phage lysozyme in the life cycle of phage T4. *Virology* 36, 387-391.
- Erickson, R. J. (1980). Bacteriophage problems in the dairy industry. *Dairy Industries* 45(3), 37, 41, 44.
- Erskine, J. M. (1970). Effect of spermine on host-cell lysis and reproduction by a lactic streptococcal bacteriophage. *Applied Microbiol.* 19, 638-642.
- Evans, A. C. (1934). Streptococcus bacteriophage and its usefulness for the identification of strains of hemolytic streptococci. *J. Bacteriol.* 27, 49-50.
- Forsen, R. (1966). Die Langmilch (pitkapiima). *Finn. J. Dairy Sci.*, 26, 1-76.
- Fuchs, P. G., Zajdel, J. and Dobrzanski, W. T. (1975). Possible plasmid nature of the determinant for production of the antibiotic nisin in some strains of *Streptococcus lactis*. *J. Gen. Microbiol.* 88, 189-192.
- Galeslout, T. E., Hassing, F. and Stadhouders, J. (1966). Differences in phage sensitivity of starters propagated in practice and in a dairy research laboratory. *XVII Int. Dairy Congr.* D, 491-498.
- Gasson, M. J. (1980). Production, regeneration and fusion of protoplasts in lactic streptococci. *FEMS Microbiol. Lett.* 9, 99-102.
- Gasson, M. J. and Davies, F. L. (1980). Prophage cured derivatives of *Streptococcus lactis* and *Streptococcus cremoris*. *Appl. Environ. Microbiol.* 40, 964-966.
- Ghuysen, J. M., Tipper, D. J. and Strominger, J. L. (1966). Enzymes that degrade bacterial cell walls. In *Methods in Enzymology*. ed. Colowick, S. P. and Kaplan, N. O. Vol. 8, p. 685-699. New York: Academic Press Inc.
- Goepfert, J. H. and Naylor, H. B. (1967). Characteristics of a lytic enzyme induced by bacteriophage infection of *Micrococcus lysodeikticus*. *J. Virol.* 1, 701-710.

Hull, R. R. (1977). Control of bacteriophage in cheese factories. *Aust. J. Dairy Technol.* 32, 65-66.

- Groman, N. B. and Suzuki, G. (1963). Quantitative study of endolysin synthesis during reproduction of lambda phages. *J. Bacteriol.* 86, 187-194.
- Groman, N. B. and Suzuki, G. (1966). Effect of spermine on lysis and reproduction by bacteriophages  $\phi$  X174,  $\lambda$ , and f2. *J. Bacteriol.* 92, 1735-1740.
- Grossowicz, N. and Ariel, M. (1963). Mechanism of protection of cells by spermine against lysozyme-induced lysis. *J. Bacteriol.* 85, 293-300.
- Gulstrom, T. J., Pearce, L. E., Sandine, W. E. and Elliker, P. R. (1979). Evaluation of commercial phage inhibitory media. *J. Dairy Sci.* 62, 208-221.
- Hargrove, R. E., McDonough, F. E. and Tittsler, R. P. (1961). Phosphate heat treatment of milk to prevent bacteriophage proliferation in lactic cultures. *J. Dairy Sci.* 44, 1799-1810.
- Hayashi, H., Araki, Y. and Ito, E. (1973). Occurrence of glucosamine residues with free amino groups in cell wall peptidoglycan from bacilli as a factor responsible for resistance to lysozyme. *J. Bacteriol.* 113, 592-598.
- Heap, H. A., Limsowtin, G. K. Y. and Lawrence, R. C. (1978). Contribution of *Streptococcus lactis* strains in raw milk to phage infection in commercial cheese factories. *N.Z. J. Dairy Sci. Technol.* 13, 16-22.
- Henning, D. R., Black, C. H., Sandine, W. E. and Elliker, P. R. (1968). Host-range studies of lactic streptococcal bacteriophages. *J. Dairy Sci.* 51, 16-21.
- Henning, D. R., Sandine, W. E. and Elliker, P. R. (1968). Chemical composition of a bacteriophage for *S. diacetilactis*. *J. Dairy Sci.* 51, 345-351.
- Holden, J. T. and van Balgooy, J. N. A. (1965). Effect of nutritional and physiological factors on the reaction between *Lactobacillus plantarum* and muramidase. *Biochem. Biophys. Res. Commun.* 19, 401-406.
- Huggins, A. R. and Sandine, W. E. (1977). Incidence and properties of temperate bacteriophages induced from lactic streptococci. *Appl. Environ. Microbiol.* 33, 184-191.
- Hunter, G. J. E. (1943). Bacteriophages for *Streptococcus cremoris*. Phage development at various temperatures. *J. Dairy Res.* 13, 136-145.
- Hunter, G. J. E. (1944). The influence of bacteriophage on the cheesemaking process. *J. Dairy Res.* 14, 294-301.
- Hunter, G. J. E. (1947). Phage resistant and phage-carrying strains of lactic streptococci. *J. Hygiene* 45, 307-312.
- Hurst, A. and Stubbs, J. M. (1969). Electron microscopic study of membranes and walls of bacteria and changes occurring during growth initiation. *J. Bacteriol.* 97, 1466-1479.
- Hurst, A. (1973). Microbial antagonism in foods. *Can. Inst. Food Sci. Technol. J.* 6, 80-90.

- Hurst, A. (1978). Nisin, its preservative effect and function in the growth cycle of the producing organism. In *Streptococci*. Society for Applied Bacteriology Symposium Series No. 7. ed. Skinner, F. A. and Quesnel, L. B. London: Academic Press.
- Hynd, J. (1976). The use of concentrated single strain starters in Scotland. *J. Soc. Dairy Technol.* 29, 39-45.
- Inouye, M. and Tsugita, A. (1968). Amino acid sequence of T2 phage lysozyme. *J. Mol. Biol.* 37, 213-223.
- Jacob, F. and Fuerst, C. F. (1958). The mechanism of lysis by phage studied with defective lysogenic bacteria. *J. Gen. Microbiol.* 18, 518-526.
- Jarvis, A. W. (1977). The serological differentiation of lactic streptococcal bacteriophage. *N.Z. J. Dairy Sci. Technol.* 12, 176-181.
- Jarvis, A. W. (1980). Phage lysins. In *New Zealand Dairy Research Institute Biennial Review*. p. 59.
- Jensen, H. B. and Kleppe, K. (1972). Studies on T4 lysozyme. Affinity for chitin and the use of chitin in the purification of the enzyme. *Eur. J. Biochem.* 26, 305-312.
- Jespersen, N. J. T. (1977). Starter problems in Cheddar cheese production. *Dairy Industries* 42(12), 41, 43, 47.
- Jones, L. J. (1956). The Jones bulk starter system. *Dairy Eng.* 73, 135-136.
- Johns, C. K. (1943). Further studies on bacteriophage in relation to Cheddar cheesemaking. *J. Dairy Res.* 13, 119-122.
- Johns, C. K. and Katznelson, H. (1941). Studies on bacteriophage in relation to Cheddar cheesemaking. *Can. J. Res.* 19, 49-51.
- Jolles, J., Dianoux, A. C., Hermann, J., Niemann, B. and Jolles, P. (1966). Relationship between the cystine and tryptophan contents of 5 different lysozymes and their heat stability and specific activity. *Biochim. Biophys. Acta.* 128, 568-570.
- Jolles, P., Saint-Blancard, J., Charlemagne, D., Dianoux, A. C., Jolles, J. and Le Earon, J. L. (1968). Comparative behaviour of six different lysozymes in the presence of an inhibitor. *Biochim. Biophys. Acta.* 151, 532-534.
- Jolles, P. *et al.* (1974). From lysozymes to chitinases: structural, kinetic and crystallographic studies. In *Lysozyme*. ed. Osserman, E. F., Canfield, R. E. and Beychok, S. London: Academic Press.
- Jones, D. (1978). Composition and differentiation of the genus *Streptococcus*. In *Streptococci*. Society for Applied Bacteriology Symposium Series No. 7. ed. Skinner, F. A. and Quesnel, L. B. London: Academic Press.
- Katz, W. (1964). Vergleichende untersuch ungen an teilchengebundenem und freiem Ts-lysozym und kristallisierung beider enzyme. *Z. Naturforsch.* 19b, 129-133.
- Keogh, B. P. (1973). Adsorption, latent period and burst size of phages of some strains of lactic streptococci. *J. Dairy Res.* 40, 303-309.

- Keogh, B. P. and Shimmin, P. D. (1974). Morphology of the bacteriophages of lactic streptococci. *Appl. Microbiol.* 27, 411-415.
- Klaenhammer, T. R., McKay, L. L. and Baldwin, K. A. (1978). Improved lysis of group N streptococci for isolation and rapid characterization of plasmid deoxyribonucleic acid. *Appl. Environ. Microbiol.* 35, 592-600.
- Knittel, M. D. (1965). Genetic homology and exchange in lactic acid streptococci. Ph.D. Thesis. Oregon State University, Corvallis, Oregon.
- Kozak, W., Rajchert-Trzpił, M., Zajdel, J. and Dobrzanski, W. T. (1973). Lysogeny in lactic streptococci producing and not producing nisin. *Appl. Microbiol.* 25, 305-308.
- Krause, R. M. and McCarty, M. (1961). Studies on the chemical structure of the streptococcal cell wall. *J. Exp. Med.* 114, 127-141.
- Law, B. A., Castanon, M. J. and Sharpe, M. E. (1976). The contribution of starter streptococci to flavour development in Cheddar cheese. *J. Dairy Res.* 43, 301-311.
- Law, B. A. and Sharpe, M. E. (1978). Streptococci in the dairy industry. In *Streptococci*. Society for Applied Bacteriology Symposium Series No. 7. ed. Skinner, F. A. and Quesnel, L. B. London: Academic Press.
- Lawrence, R. C. (1978). Action of bacteriophages on lactic acid bacteria: consequences and protection. *N.Z. J. Dairy Sci. Technol.* 13, 129-136.
- Lawrence, R. C. and Pearce, L. E. (1968). The case against the use of unpaired single strain starters. *N.Z. J. Dairy Sci. Technol.* 3, 137-139.
- Lawrence, R. C. and Pearce, L. E. (1972). Cheese starters under control. *Dairy Industries* 37, 73-78.
- Lawrence, R. C., Thomas, T. D. and Terzaghi, B. E. (1976). Reviews of the progress of dairy science: Cheese starters. *J. Dairy Res.* 32, 141-193.
- Lawrence, R. C., Heap, H. A., Limsowtin, G. and Jarvis, A. W. (1978). Cheddar cheese starters: Current knowledge and practices of phage characteristics and strain selection. *J. Dairy Sci.* 61, 1181-1191.
- Lawrence, R. C. and Thomas, T. D. (1979). The fermentation of milk by lactic acid bacteria. In *Microbial Technology Current State, Future Prospects*. Society for General Microbiology Symposium 29. ed. Bull, A. T., Ellwood, D. C. and Ratledge, C. Cambridge: Cambridge University Press.
- Lees, G. J. and Jago, G. R. (1976). Acetaldehyde: an intermediate in the formation of ethanol from glucose by lactic acid bacteria. *J. Dairy Res.* 43, 63-73.
- Lewis, J. E. (1956). A new approach to the problem of phage control during the production of commercial cheese starters. *J. Soc. Dairy Technol.* 9, 123-128.
- Limsowtin, G. K. Y. and Terzaghi, B. E. (1976). Agar medium for the differentiation of 'fast' and 'slow' coagulating cells in lactic streptococcal cultures. *N.Z. J. Dairy Sci. Technol.* 11, 65-66.

- Limsowtin, G. K. Y., Heap, H. A. and Lawrence, R. C. (1977). A multiple starter concept for cheesemaking. *N.Z. J. Dairy Sci. Technol.* 12, 101-106.
- Limsowtin, G. K. Y. and Terzaghi, B. E. (1977). Characterisation of bacterial isolates from a phage-carrying culture of *Streptococcus cremoris*. *N.Z. J. Dairy Sci. Technol.* 12, 22-28.
- Limsowtin, G. K. Y., Heap, H. A. and Lawrence, R. C. (1978). Heterogeneity among strains of lactic streptococci. *N.Z. J. Dairy Sci. Technol.* 13, 1-8.
- Lister, J. (1878). On the lactic fermentation and its bearing on pathology. *Trans. Pathol. Soc.* 29, 425-465.
- Logardt, I.-M and Neujahr, H. Y. (1975). Lysis of modified walls from *Lactobacillus fermentum*. *J. Bacteriol.* 124, 73-77.
- London, J. (1976). The ecology and taxonomic status of the lactobacilli. *Ann. Rev. Microbiol.* 30, 279-301.
- Lowrie, R. J. (1974). Lysogenic strains of Group N streptococci. *Appl. Microbiol.* 27, 210-217.
- McDonald, I. J. (1971). Filamentous forms of *Streptococcus cremoris* and *Streptococcus lactis*. Observations on structure and susceptibility to lysis. *Cand. J. Microbiol.* 17, 897-902.
- McKay, L. L. and Baldwin, K. A. (1973). Induction of prophage in *Streptococcus lactis* C2 by ultraviolet irradiation. *Appl. Microbiol.* 25, 682-684.
- Mandelstam, M. and Strominger, J. L. (1961). On the structure of the cell wall of *Staphylococcus aureus* (Copenhagen). *Biochem. Biophys. Res. Commun.* 5, 466-471.
- Markert, A. and Zillig, W. (1965). Studies on the lysis of *Escherichia coli* C by bacteriophage  $\phi$ X174. *Virology* 25, 88-97.
- Marshall, R. J. and Berridge, N. J. (1976). Selection and some properties of phage-resistant starters for cheesemaking. *J. Dairy Res.* 43, 449-458.
- Maxted, W. R. (1957). The active agent in nascent phage lysis of streptococci. *J. gen. Microbiol.* 16, 584-595.
- Meanwell, L. J. and Thompson, N. (1959). The influence of rennet in bacteriophage infection in the cheese vat. *J. Appl. Bacteriol.* 22, 281-286.
- Meyer, K., Palmer, J. W., Thompson, R. and Khorazo, D. (1936). On the mechanism of lysozyme action. *J. Biol. Chem.* 113, 479-486.
- Moo-Penn, W. and Wiesmeyer, H. (1969). The isolation and characterization of coliphage N<sub>20</sub>F and properties of induced lytic enzyme. *Biochim. Biophys. Acta.* 178, 318-329.
- Mou, L., Sullivan, J. J. and Jago, G. R. (1976). Autolysis of *Str. cremoris*. *J. Dairy Res.* 43, 275-282.
- Moustafa, H. H. and Collins, E. B. (1968a). Role of galactose or glucose-1-phosphate in preventing the lysis of *Streptococcus diacetylactis*. *J. Bacteriol.* 95, 592-602.



- Moustafa, H. H. and Collins, E. B. (1968b). Molar growth yield of certain lactic acid bacteria as influenced by autolysis. *J. Bacteriol.* 96, 117-125.
- Mukai, F., Streisinger, G. and Miller, B. (1967). The mechanism of lysis in phage T4-infected cells. *Virology* 33, 398-402.
- Mullan, W. M. A. (1977). The effects of temperature on the interactions of the lactic streptococci and their bacteriophages. M.Sc. thesis, University College, Cork, Ireland.
- Mullan, W. M. A. (1979). Lactic streptococcal bacteriophage enumeration. A review of factors affecting plaque formation. *Dairy Industries* 44, (7) 11, 13, 15.
- Mullan, W. M. A. and Walker, A. L. (1979). An agar medium and a simple streaking technique for the differentiation of the lactic streptococci. *Dairy Industries* 44 (6), 13, 17.
- Mullan, W. M. A., Daly, C. and Fox, P. F. (1981a). Effects of temperature on the interaction of phage M18(1) and *Streptococcus lactis* m18. *Milchwissenschaft* 36, 288-290.
- Mullan, W. M. A., Daly, C. and Fox, P. F. (1981b). Effect of cheesemaking temperatures on the interactions of lactic streptococci and their phages. *J. Dairy Res.* 48, 465-471.
- Murphy, J. S. (1957). A phage-associated enzyme of *Bacillus megaterium* which destroys the bacterial cell wall. *Virology* 4, 563-581.
- Murphy, J. S. (1961). An agent derived from *B. megaterium* phage G which dissolves the bacterial cell wall. *Virology* 11, 510-513.
- Muskowitz, G. J., Staehler, G. and La Belle, G. (1979). Modification of the New Zealand culture concept in the manufacture of Cheddar cheese in the United States. *J. Dairy Sci.* 61 (Suppl. 1), 116 (Abst.).
- Natkin, E. (1962). A study of bacteriophage-induced lysis of *Carynebacterium diphtheriae*. M.A. Thesis, University of Washington, Seattle.
- Naylor, J. and Czulak, J. (1956). Host-range relationships of cheese starter organisms. II. Effect of phage activity on heterologous strains of lactic streptococci. *J. Dairy Res.* 33, 126-130.
- Nelson, F. E., Harriman, L. A. and Hammer, B. W. (1939). Slow acid production by butter cultures. *Iowa Agr. Expt. Sta. Research Bull.* 256.
- Nichols, A. A. and Wolf, J. Z. (1945). Observations on cheese starters with reference to bacteriophage and the phage-organism relationship of strains isolated. *J. Dairy Res.* 14, 81-93.
- Neujahr, H. Y. (1966). Transport of B-vitamins in microorganisms, V. Comparative studies on the A.T.P.-hydrolyzing activities of cell fractions obtained from thiamine sufficient and thiamine deficient cells of *L. fermenti*. *Acta. Chem. Scand.* 20, 1518-1528.
- Neujahr, H. Y., Borstad, B. A. and Logardt, I-M. (1973). Factors affecting the resistance of *Lactobacillus fermenti* to lysozyme. *J. Bacteriol.* 116, 694-698.

Pearce, L. E. and Lowrie, R. J. (1974). Host controlled modification and restriction of bacteriophage host range by strains of *Streptococcus cremoris* and *Str. lactis* used as starters in cheesemaking. *XIX Inter. Dairy Congr.* 1E, 410-411.

- Nyiendo, J. A. (1974). Studies on host range, fine structure, and nucleic acids of lactic streptococcus bacteriophages. Ph.D. Thesis, Oregon State University, Corvallis, Oregon, U.S.A.
- Oram, J. D. (1965). Studies on the effects of lactoperoxidase and of bacteriophage on lactic acid streptococci. Ph.D. Thesis, University of Reading.
- Oram, J. D. (1971). Isolation and properties of a phage receptor substance from the plasma membrane of *Streptococcus lactis* ML3. *J. gen. Virol.* 13, 59-71.
- Oram, J. D. and Reiter, B. (1965). Phage-associated lysins affecting group N and group D streptococci. *J. gen. Microbiol.* 40, 57-69.
- Oram, J. D. and Reiter, B. (1968). The adsorption of phage to group N streptococci. The specificity of adsorption and the location of phage receptor substances in cell wall and plasma-membrane fractions. *J. gen. Virol.* 3, 103-119.
- Ou, L. T. and Marquis, R. E. (1970). Electro-mechanical interactions in cell walls of Gram-positive cocci. *J. Bacteriol.* 101, 92-101.
- Pearce, L. E. (1978). The effect of host-controlled modification on the replication rate of a lactic streptococcal bacteriophage. *N.Z. J. Dairy Sci. Technol.* 13, 166-171.
- Pearce, L. E., Limsowtin, G. K. Y. and Crawford, A. M. (1970). Bacteriophage multiplication characteristics in Cheddar cheesemaking. *N.Z. J. Dairy Sci. Technol.* 5, 141-145.
- Pecsok, R. L. and Saunders, D. (1968). On the mechanism of gel chromatography of inorganic salts. *Separ. Sci.* 3, 325-355.
- Pette, J. W. (1953). The action of bacteriophages on lactic acid bacteria. *13th International Dairy Congr.* 3, 1180-1184.
- Porter, M. C. (1972). Applications of membranes to enzyme isolation and purification. In *Enzyme Engineering*. ed. Wingard, L. B. London: Interscience Publishers.
- Pirie, A. (1939). Chemical changes occurring during lysis of *Bacillus coli* by bacteriophage. *Brit. J. Expt. Path.* 20, 99-108.
- Potter, N. N. and Nelson, F. E. (1952). Effects of calcium on proliferation of lactic streptococcus bacteriophage. Studies on plaque formation with a modified plating technique. *J. Bacteriol.* 64, 105-111.
- Potter, N. N. and Nelson, F. E. (1953). Role of calcium and related ions in proliferation of lactic streptococcus bacteriophage. *J. Bacteriol.* 66, 508-516.
- Prouty, C. C. and Parisot, D. (1957). Observations on the numbers of virus-resistant mutants developing in the host-virus system of the lactic streptococci. *J. Dairy Sci.* 40, 1193-1197.
- Ralston, D. J., Baer, B., Lieberman, M. and Krueger, A. P. (1955). Virolysin: a virus induced lysin from staphylococcal phage lysates. *Proc. Soc. Exp. Biol. Med.* 89, 502-507.

Sandine, W. E. (1977). New techniques in handling lactic cultures to enhance their performance. *J. Dairy Sci.* 60, 822-828.

- Ralston, D. J., Baer, B., Lieberman, M. and Krueger, A. P. (1957). Lysis from without of *S. aureus* K1 by the combined action of phage and virolysin. *J. gen. Physiol.* 41, 343-358.
- Ralston, D. J., Baer, B., Lieberman, M. and Krueger, A. P. (1961). Virolysin: a virus-induced lysin. Its appearance and function in phage-infected staphylococci. *J. gen. Microbiol.* 24, 313-325.
- Reddy, M. S., Vedamuthu, E. R., Washam, C. J. and Reinbold, G. W. (1972). Agar medium for differential enumeration of lactic streptococci. *Appl. Microbiol.* 24, 947-952.
- Reiter, B. (1949). Lysogenic strains of lactic streptococci. *Nature* 164, 667-668.
- Reiter, B. (1973). Some thoughts on cheese starters. *J. Soc. Dairy Technol.* 26, 2-15.
- Reiter, B. and Møller-Madsen, A. (1963). Reviews of the progress of dairy science. Section B. Cheese and butter starters. *J. Dairy Res.* 30, 419-456.
- Reiter, B. and Oram, J. D. (1963). Group N streptococcal phage lysin. *J. gen. Microbiol.* 32, 29-32.
- Richardson, G. H., Hong, G. L. and Ernstrom, C. A. (1980). Defined single strains of lactic streptococci in bulk culture for Cheddar and Monterey cheese manufacture. *J. Dairy Sci.* 63, 1981-1986.
- Robertson, P. S. (1960). Phage resistance in pure strains of cheese starter streptococci. *J. Dairy Res.* 27, 161-164.
- Robertson, P. S. (1966a). Bulk starter preparation in New Zealand cheese factories. *17th Inter. Dairy Congr. D:2*, 439-446.
- Robertson, P. S. (1966b). Starter pairing. *N.Z. J. Dairy Sci. Technol.* 1, 15-16.
- Rogers, H. J., Perkins, H. R. and Ward, J. B. (1980). *Microbial cell walls and membranes*. London: Chapman and Hall.
- Rogosa, M. (1970). Characters used in the classification of lactobacilli. *Inter. J. Syst. Bacteriol.* 20, 519-533.
- Sanders, M. E. and Klaenhammer, T. R. (1980). Restriction and modification in group N streptococci: effect of heat on development of modified lytic bacteriophage. *Appl. Environ. Microbiol.* 40, 500-506.
- Sandine, W. E., Elliker, P. R. and Anderson, A. W. (1959). Taxonomic study of high carbon dioxide-producing lactic acid streptococci isolated from mixed strain starter cultures. *J. Dairy Sci.* 44, 799-808.
- Saxelin, M-L., Nurmiaho, E-L., Korhola, M. P. and Sundman, V. (1979). Partial characterisation of a new C3-type capsule-dissolving phage of *Streptococcus cremoris* C. *Cand. J. Microbiol.* 25, 1182-1187.
- Schleifer, K. H. and Kandler, O. (1967). Zur chemischen Zusammensetzung der Zellwand der Streptokokken. II. Die Aminosäuresequenz des mureins von *Streptococcus lactis* und *cremoris*. *Arch. Mikrobiol.* 57, 365-381.

- Schleifer, K. H. and Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bact. Revs.* 36, 407-477.
- Sherman, J. M. (1937). The streptococci. *Bact. Rev.* 1, 3-97.
- Shew, D. I. (1949). Effect of calcium on the development of streptococcal bacteriophages. *Nature* 164, 492-493.
- Shockman, G. D., Thompson, J. S. and Conover, J. M. (1967). The autolytic system of *Streptococcus faecalis*. II. Partial characterisation of the autolysin and its substrate. *Biochemistry* 6, 1054-1065.
- Shockman, G. D., Thompson, J. S. and Conover, H. J. (1968). The relationship of autolysin to lysozyme sensitivity of *Streptococcus faecalis* 9790. In *Microbial protoplasts, spheroplasts and L-forms*. ed. Guze, L. B. Baltimore: Williams and Wilkins Co.
- Shungu, D. L., Cornett, J. B., Shockman, G. D. (1980). Lipids and lipoteichoic acid of autolysis-defective *Streptococcus faecium* strains. *J. Bacteriol.* 142, 741-746.
- Siegel, J. L., Hurst, S. F., Liberman, E. S., Coleman, S. E. and Bleiweis, A. S. (1981). Mutanolysin-induced spheroplasts of *Streptococcus mutans* are true protoplasts. *Infect. Immun.* 9, 60-67.
- Spackman, D. H., Stein, W. H. and Moore, S. (1958). Automatic recording apparatus for use in the chromatography of amino acids. *Anal. Chem.* 30, 1190-1206.
- Sozzi, T., Poulin, J. M., Maret, R. and Pousaz, R. (1978). Isolation and some characteristics of phages of ropy strains of *Streptococcus lactis*. *Milchwissenschaft* 44, 349-352.
- Stadhouders, J. (1976). Control of starter activity and the use of starter concentrates. *Nord. Mejeri-tidsska.* 42 (6) 191-208.
- Stark, J. G. and Wallace, H. G. (1969). *Chemistry Data Book*. London: John Murray.
- Strominger, J. L. and Ghuyssen, J. -M. (1968). Mechanism of enzymatic bacteriolysis. *Science* 156, 213-221.
- Tabor, C. W. (1962). Stabilization of protoplasts and spheroplasts by spermine and other polyamines. *J. Bacteriol.* 83, 1101-1111.
- Terzaghi, B. E. (1976). Morphologies and host sensitivities of lactic streptococcal phages from cheese factories. *N.Z. J. Dairy Sci. Technol.* 11, 155-163.
- Terzaghi, B. E. and Sandine, W. E. (1975). Improved medium for lactic streptococci and their bacteriophages. *Appl. Microbiol.* 29, 807-813.
- Thibodeau, R. and Rendak, A. (1962). Le contrôle du bactériophage dans la fabrication du fromage 24 heures par jour. *16th Int. Dairy Congr.* B, 757-764.

- Thomas, T. D., Jarvis, B. D. W. and Skipper, N. A. (1974). Localization of proteinase(s) near the cell surface of *Streptococcus lactis*. *J. Bacteriol.* 118, 329-333.
- Tourville, D. R. and Johnstone, D. B. (1966). Lactic streptococcal phage-associated lysin. 1. Lysis of heterologous lactic streptococci by a phage-induced lysin. *J. Dairy Sci.* 49, 158-162.
- Tourville, D. R. and Tokuda, S. (1967). Lactic streptococcal phage-associated lysin. 11. Purification and characterization. *J. Dairy Sci.* 50, 1019-1024.
- Tsaneva, K. P. (1976). Electron microscopy of virulent phages for *Streptococcus lactis*. *Appl. Environ. Microbiol.* 31, 590-601.
- Tsugita, A. (1971). Phage lysozyme and other lytic enzymes. In *The Enzymes*. 2nd Edition, Vol. 5. ed. Boyer, P. D. London: Acad. Press Inc.
- Tsugita, A. and Inouye, M. (1968). Complete primary structure of phage lysozyme from *Escherichia coli* T4. *J. Mol. Biol.* 37, 201-212.
- Tsugita, A., Inouye, M., Terzaghi, E. and Streisinger, G. (1968). Purification of bacteriophage T4 lysozyme. *J. Biol. Chem.* 243, 391-397.
- von Geis, A. and Teuber, M. (1981). The bacteriocins of lactic streptococci. Paper presented at the International Circle of Dairy Research Leaders Conference: Genetic Modification of starter cultures. National Institute for Research in Dairying, Shinfield.
- Walker, A. L., Mullan, W. M. A. and Muir, M. E. (1981). A survey of bulk starter production in the cheese industry. *J. Society Dairy Technol.* 34, 78-84.
- Wallinder, I. B. and Neujahr, H. Y. (1971). Cell wall and peptidoglycan from *Lactobacillus fermenti*. *J. Bacteriol.* 105, 918-926.
- Weaver, G. L., Kroger, M. and Katz, F. (1977). Deaminated chitin affinity chromatography: a method for the isolation purification and concentration of lysozyme. *J. Food Sci.* 42, 1084-1087.
- Welker, N. E. (1967). Purification and properties of a thermophilic bacteriophage lytic enzyme. *J. Virology* 1, 617-625.
- White, J. R. and Hurst, A. (1968). The location of nisin in the producer organism *Streptococcus lactis*. *J. Gen. Microbiol.* 53, 171-179.
- Whitehead, H. R. (1953). Bacteriophage in cheese manufacture. *Bact. Rev.* 17, 109-123.
- Whitehead, H. R. and Cox, G. A. (1934). Observations on sudden changes in the rate of acid formation in milk by cultures of lactic streptococci. *J. Dairy Res.* 5, 197-207.
- Whitehead, H. R. and Cox, G. A. (1935). The occurrence of bacteriophage in cultures of lactic streptococci. *N.Z. J. Sci. Technol.* 16, 319-320.

Whitehead, H. R. and Cox, G. A. (1936). Bacteriophage phenomena in cultures of lactic streptococci. *J. Dairy Res.* 7, 55-62.

Whitehead, H. R. and Hunter, G. J. E. (1945). Bacteriophage infection in cheese manufacture. *J. Dairy Res.* 14, 64-80.

Whitehead, H. R. and Hunter, G. J. E. (1947). Bacteriophage in cheese manufacture. Contamination from farm equipment. *J. Dairy Res.* 15, 112-120.

Whitehead, H. R., Hunter, G. J. E. and Cox, G. A. (1952). Inhibition of bacterial growth by bacteriophage as distinct from lytic action. *J. gen. Microbiol.* 6, 21-29.

Whitehead, H. R., East, A. and McIntosh, L. (1953). The so-called 'nascent' bacteriophage phenomenon. *J. Dairy Res.* 20, 60-64.

Wolf, J. Z., Nichols, A. A. and Ineson, P. J. (1946). Mists containing hypochlorite as germicides in the destruction of airborne bacteriophage attacking lactic streptococci. *J. Dairy Res.* 14, 291-315.

Zinder, N. D. (1965). RNA phages. *Ann. Rev. Microbiol.* 19, 455-472.

Zehren, V. L. and Whitehead, H. R. (1954). Growth characteristics of streptococcal phages in relation to cheese manufacture. *J. Dairy Sci.* 37, 209-219.